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14. ABSTRACT

Chromosomal rearrangements and changes in copy number at various genomic loci are hallmarks of cancer cells and may be very early steps in tumorigenesis. The origins of genomic insults are poorly understood and this proposal aims to characterize one potential source of genomic instability, inappropriate DNA re-replication. In a normal eukaryotic cell cycle, the chromosomal DNA of a cell is replicated once, and only once, during S phase to ensure that each daughter cell receives exactly one complement of genomic material. By perturbing the regulation of several proteins involved in replication initiation, our laboratory has been able to conditionally induce varying amounts of re-replication in yeast cells. In the prior reporting period we demonstrated that re-replication induces a rapid and significant decrease in cell viability and a cellular DNA damage response. Strikingly, we have observed DNA damage in the absence of a classical replication stress response. In this reporting period we have demonstrated that re-replication leads to genome instability, in particular gene duplication. This is the first experimental evidence that re-replication might contribute to gene amplification and thus tumorigenesis.

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Introduction

Genomic instability is common in breast cancer cells and can lead to loss of heterozygosity (Taback et al., 2003), gene amplifications (Lopez-Guerrero et al., 2003) and other genomic rearrangements. By inducing genomic alterations, genomic instability may promote carcinogenesis or make cancers more resistant to treatment. Currently, the source of genomic instability is unknown and this work aims to characterize one potential source of genomic instability, inappropriate DNA re-replication. In a normal eukaryotic cell cycle, the chromosomal DNA of a cell is replicated once, and only once, during S phase to ensure that each daughter cell receives exactly one complement of genomic material. By perturbing the regulation of several proteins involved in replication initiation, our laboratory has been able to conditionally induce varying amounts of re-replication in yeast cells. Effectively, cells enter, but do not complete, a second S phase (Nguyen et al., 2001), because only part of the genome re-replicates. The research supported by this grant is focused on understanding the consequences of such re-replication. Specifically, we have shown that re-replication leads to DNA damage and cell inviability. In this reporting period, we have demonstrated that re-replication leads to genomic instability, in particular gene duplication.

Body

During the period from March 24th, 2005 to March 23rd, 2006, significant progress was made on a number of the tasks described in the initial application for this grant, particularly task 6. In addition, we characterized a number of experimental systems that have been, and will be, used to complete these tasks and published this characterization. I am a first author on this paper that was published in February 2006 in the journal Molecular Biology of the Cell (Appendix 1, Green et al, 2006). I was also asked to give a talk at the Eukaryotic DNA Replication Meeting at Cold Spring Harbor Laboratory, New York on September 9th, 2005 where I presented work done with the support of this grant.

The overall purpose of the grant was to study the consequences of re-replication of cellular DNA. During a normal cell cycle, DNA replication is tightly controlled such that the genome is replicated once and only once before each mitosis. Loss of replication control has been proposed to be a source of the genomic instability that is associated with tumorigenesis. Our laboratory, and others, has elucidated many of the mechanisms that prevent re-replication from occurring. In doing so, we have established a yeast system with which we can induce re-replication in a population of cells arrested in metaphase. The work that has been supported by this grant is focused on understanding the consequences of this inappropriate DNA replication.

Task 1: Confirm that re-replication induces a cellular DNA damage response.

In the previous reporting period, we completed task 1 of the initial grant application, which was to confirm that there was a DNA stress response as a consequence of re-replication. We additionally expanded our efforts in relation to this aim due to the surprising discovery that re-replication leads to a DNA damage response seemingly in the absence of the replication stress response. Much of this work was published in a manuscript described in the prior annual report (Green and Li, 2005).

Task 2: Establish whether pre-RC reformation, re-initiation or re-elongation induces the DNA damage response.

In task 2 of the initial grant application, we proposed to determine whether inappropriate pre-RC formation, re-initiation or re-elongation is the cause of the DNA damage observed when re-replication is induced. In the prior annual report, we demonstrated that inappropriate pre-RC formation is not sufficient to lead to DNA damage. We proposed to use hydroxyurea (HU), a ribonucleotide reducatase inhibitor, to reduce re-elongation while still allowing re-initiation to occur in order to determine which of those two steps resulted in DNA damage. However, we have demonstrated that, surprisingly, re-initiation is markedly reduced when cells are treated with HU (data not shown) and thus we cannot prevent re-elongation without reducing re-initiation. We do not have an explanation for this result other than to note that the Brewer lab has reported at meetings that their genomic DNA replication assays suggest that, contrary to expectation, initiation in S phase is in fact reduced in the presence of HU (personal

communication, B. Brewer). Although we have accomplished the first portion of task 2, we are thus unable to fully complete task 2 as described.

Task 3: Examine the structure of the DNA lesions induced by re-replication with electron microscopy.

My next task (task 3) was to use electron microscopy to determine the nature of DNA lesions induced by re-replication. Since re-replication initiation is required for DNA damage, as described above, it is likely that electron microscopy will be very useful to visualize the actual DNA lesions induced by re-replication. As described in the prior annual report, initial attempts to conduct these technically difficult experiments in our laboratory proved to be unfruitful. Consequently, we established collaboration with Dr. Jose Sogo to help us complete task 3. Dr. Sogo is the world's foremost expert on studying DNA lesions using electron microscopy (Sogo et al., 2002) and has agreed to teach me his electron microscopy technique. Although we had hoped to begin this collaboration during this reporting period, Dr. Sogo is currently in transition between two institutes and he is without access to an electron microscope. We are currently exploring the feasibility of hosting him at UCSF or at UC Berkeley for a few weeks to teach me his technique.

Task 4: Search for double stranded break zones induced by re-replication.

We are very interested in determining where in the genome re-replication induced DNA damage occurs. If there are specific regions of increased damage we will attempt to correlate them with chromosomal features such as centromeres, cohesin binding sites and origins of re-replication. In the manuscript published during this reporting period (Green et al, 2006) we determine, on a genome wide level, the location of origins of DNA replication (Appendix 1, Figure 2). These data will be needed to correlate any regions of DNA damage with regions of re-replication.

Task 4 proposed the use of pulsed field gel electrophoresis to look for fragile zones where chromosomal breakage occurs as a consequence of re-replication. Despite several attempts and consultation with researchers who have used this technique, we have been unable to detect fragile zones (data not shown). The absence of a signal does not in any way demonstrate that these zones do not exist, as a limitation of this assay is that chromosomes currently re-replicating run aberrantly on the gel. Even if many molecules were broken in the same location, variable extents of re-replication would result in them running at different locations on the gel. Consequently, we have decided to use another assay to look for regions of preferential damage.

Chromatin immunoprecipitation can be used to purify DNA that is bound by a protein of interest. It has been shown that Ddc2, a DNA damage response protein, localizes to sites of DNA damage (Melo et al, 2001) and I have demonstrated that Ddc2 sub-nuclear foci form when re-replication is induced (Green and Li, 2005). Dr. Katsu Shirahige is an expert at hybridizing DNA isolated using chromatin immunoprecipitation to DNA microarrays (Katou et al, 2003). This technology allows the precise

determination of DNA binding sites of a protein across entire chromosomes. Hybridizing DNA immunoprecipitated using antibodies to Ddc2 will allow us to determine where DNA damage is occurring as damaged DNA will be bound to Ddc2 and thus enriched in the precipitated DNA.

We have established collaboration with Dr. Shirahige and have received strains and plasmids from his lab to conduct our experiments. We will induce re-replication and perform the chromatin immunoprecipitation. We will send Dr. Shirahige the immunoprecipitated DNA and his lab will amplify, label and hybridize the DNA to microarrays. I am currently preparing DNA to send to his lab and we believe that we will be able to address the question proposed in task 4 using this new technique.

Task 5: Establish whether re-replication leads to loss of heterozygosity.

Tasks 5 and 6 are focused on determining the long term consequences of re-replication on the stability of the genome. However, as we reported in the prior annual report, extensive re-replication leads to signification cell inviability (Green and Li, 2005). In order to study potential consequences of re-replication, we needed to establish a strain in which the cell death was reduced. We did this by perturbing two, rather than three, mechanisms that block re-replication. We have demonstrated that making these changes does result in reduced re-replication, in fact under some conditions, we are able to observe re-replication primarily from a single origin of replication.

The strain in which re-replication occurs primarily from a single origin of DNA replication will be used for tasks 5 and 6. Since demonstrating that re-replication occurs in this strain was essential before using it to study genomic instability, I delayed work on some of the tasks in my initial proposal in order to prepare this manuscript for publication. The manuscript has now been published in Molecular Biology of the Cell (Green et al, 2006) and is attached as Appendix 1 to this annual report.

In this study, we used microarray comparative genomic hybridization (CGH; Appendix 1, Figure 1) to provide a more comprehensive and detailed analysis of re-replication. Among other things, we demonstrate that re-replication can be induced within S phase (Appendix 1, Figure 3). We also show that it differs in amount and location from re-replication in G2/M phase, illustrating the dynamic nature of DNA replication controls (Appendix 1, Figures 2, 4 and 5). We note that re-replication occurs more readily during S phase, which could increase the likelihood that re-replication might occur in cells with fewer perturbations to cell cycle controls of DNA replication.

Finally, we show that very limited re-replication can be detected by microarray CGH when only two replication proteins are deregulated, suggesting that the mechanisms blocking re-replication are not redundant (Appendix 1, Figures 5, 6 and 7). The application of a more sensitive assay to re-replication allowed us to observe re-replication that had previously been undetectable. It seems more likely, therefore, that although cancer cells have not been observed to re-replicate, a more sensitive assay might be able to detect limited re-replication. Therefore we propose that eukaryotic re-replication at

levels below current detection limits may be more prevalent and a greater source of genomic instability than previously appreciated.

We are thus prepared with the required strains to conduct task 5, but exciting results on task 6 have led us to focus on that task and switch the order of execution of tasks 5 and 6.

Task 6: Determine if re-replication promotes gene amplification.

In the initial grant application, we proposed to use a cassette of the ADH4 and CUP1 genes to select for gene amplification events. Although use of this system has been published before, there are a number of disadvantages of using this technique. The genetic modifications needed to use this system are complicated and time consuming to introduce and make the cells quite sick. Additionally, up to 4 to 7 copies of the cassette are required to grow under selective conditions, so this assay cannot detect the earliest and primary amplification event when a chromosomal region is first stably amplified from one to two copies. Consequently we decided to investigate whether we could develop a better system.

During this reporting period, we have developed a new assay (Figure 1) that allows us to detect primary amplification events. In addition, the assay is versatile and allows the detection of other types of genomic rearrangements that result in heritable duplication of a chromosomal segment (Figure 1). We define a gene duplication event as one that results in two or more copies of a gene in a single cell, and a gene amplification as a gene duplication event that results in the two copies on a single DNA molecule. The assay is adapted from a colony color sectoring assay that can distinguish cells in a colony that have either 1 or ≥2 copies of ade3-2p, a hypomorphic allele of ADE3, based on whether the cells are pink or red, respectively (Koshland et al, 1985).

We can monitor the duplication of any locus in a cell lineage by inserting the ade3-2p reporter at that locus and looking for pink colonies with red sectors. Since all cells in a colony are derived from a single starting cell, a red sector indicates that one cell in the lineage has undergone a heritable increase in gene copy number. The greater the width of the sector, the earlier in the cell lineage this heritable change occurred. In preliminary experiments, we have inserted a single copy of ade3-2p approximately 5 kb centromere-distal to ARS317 in the strain that re-replicates primarily from ARS317 (Figure 1 and Appendix 1, Figure 7). After transiently inducing re-replication at a G2/M phase arrest with galactose induction of Δntcdc6-2A in liquid culture, we plated the cells and monitored the number of colonies with red sectors.

To identify heritable changes that occurred shortly after the re-replication insult, we monitored the frequency of sectors that comprise a half, quarter, or an eighth of the colony since these sectors reflect a single heritable event that occurs within the first three cell divisions in the colony. We observed that transient re-replication caused a 70-fold increase in the number of sectored colonies, indicating that re-replication triggers a robust increase in heritable gene duplication events that are easily detectable by this assay

(Figure 2). The increase in sectoring frequency was dependent on galactose-induced expression of Δntcdc6-2A, and was not observed in a control strain that did not harbor this deregulated CDC6 allele. Duplication of the reporter is also significantly reduced when ARS317 is deleted or when the reporter is moved away from ARS317 onto chromosome VI. Preliminary results also indicate that mutations that disrupt G1 progression (cdc4), replication initiation (cdc6, cdc7), DNA polymerases (cdc2, cdc17), DNA ligase (cdc9), telomere maintenance (cdc13) and mitosis (cdc15) generate gene duplication at frequencies below 10-3 (data not shown), significantly less than the re-replicating strain.

In the original task 6 we proposed to analyze the structure of putative gene duplications by a battery of assays (Q-PCR, microarray CGH, PFGE, and Southern blotting). We have applied these same assays to a number of colonies containing gene duplications of the ade3-2p cassette. We now have preliminary results that: (1) confirm the accuracy of this colony assay in detecting gene duplications; (2) demonstrate our ability to perform high throughput microarray CGH on hundreds of yeast isolates; (3) suggest that re-replication but not other cell cycle or chromosomal perturbations, can induce gene duplications; (4) suggest that re-replication may specifically induce internal gene duplications (possibly representing a primary gene amplification event); and (5) suggest ways to refine our screen to focus on internal gene duplications.

For further genomic analysis of isolates identified by the sectoring assay, we have adapted high throughput microarray techniques developed by Christine Guthrie's lab here at UCSF. In our first trial we performed microarray CGH analyses on 49 isolates within four days (from inoculation of cultures to complete analysis of microarray data). 43 had indeed duplicated the reporter, confirming the reliability of the sectoring assay. Of these, 39 had an extra copy of chromosome III, and 4 had an extra centromeric fragment of chromosome III. Whether the duplication of chromosome III is due to re-replication of the entire chromosome (which is only 350 kb in length) or, more interestingly, to re-replication induced chromosome nondisjunction, may be distinguished if ARS317 can still reinitiate after being moved to a large chromosome. Disomy due to full chromosome rereplication from a single origin would presumably occur at lower frequencies for larger chromosomes. We suspect the relocated ARS317 will still re-initiate, since Steve Bell's lab has successfully moved a re-initiating origin to a new chromosomal locus and still observed reinitiation (Tanny et al, 2006). We are in the progress of testing whether ARS317 can re-replicate when moved to chromosome 4, one of the largest chromosomes in the yeast genome.

Interestingly, one of the 49 strains analyzed by CGH contained an acentric duplication of an internal fragment flanked by Ty (retrotransposition) elements on chromosome VII (Figure 3). Such internal duplications may represent primary gene amplification events, especially if further structural analysis confirms that these duplications are tandemly arrayed. Transposons were observed at break points of many of the chromosomal rearrangements we have studied (data not shown) and in budding yeast that were analyzed by the Botstein lab (Dunham et al, 2002). Hence, we hypothesize that we may observe internal duplications most readily when re-initiation is

induced between two Ty elements. ARS317 is not in such a position so, as stated above, we are moving it and the ade3-2p reporter gene to other chromosomal loci.

This reporting period we have demonstrated that re-replication does, in fact, lead to genome instability, gene duplication and possibly gene amplification. We have established the strains and assays required to conclusively determine whether gene amplification is a consequence of re-replication, and look forward to conducting those experiments.

Key Research Accomplishments

Key accomplishments in this reporting period:

We have fully characterized strains in which re-replication is very limited

We have fully characterized the location and extend of re-replication in numerous re-replicating strains

We have demonstrated that re-replication can occur in S phase

We have adapted a single cell assay to screen for gene duplication events

We have demonstrated that re-replication leads to a significant increase in gene duplication events and have specifically:

Confirmed the accuracy of this colony assay in detecting gene duplications
Generated a protocol to perform high throughput microarray CGH
Suggested that re-replication but not other cell cycle or chromosomal perturbations, can induce gene duplications
Suggested that re-replication may specifically induce internal gene duplications (possibly representing a primary gene amplification event)
Suggested ways to refine our screen to focus on internal gene duplications.

Key accomplishments in the prior reporting period:

I have demonstrated that re-replication leads to DNA damage and specifically, I have shown that:

Extensive re-replication leads to significant cell inviability
Re-replication leads to a *RAD9* and *RAD53* dependent metaphase arrest
Ddc2-GFP foci form in the presence of re-replication
Re-replication leads to Rad53p phosphorylation in a RAD9 dependent manner
Direct evidence of DNA double strand breaks can be observed after re-replication

The DNA damage response due to re-replication requires replication initiation

We have established strains in which re-replication is very limited – largely occurring from a single origin of DNA replication

Limited re-replication from these strains also induces a DNA damage response

Finally, I have demonstrated that cells are capable of surviving limited and transient re-replication, setting the stage for studying genomic instability in these cells

Reportable Outcomes

This following are reportable outcomes for this reporting period:

We have published a second manuscript in Molecular Biology of the Cell describing some of the work supported by this grant (Green et al, 2006, Appendix 1).

I presented this work in a talk at the Eukaryotic DNA Replication Meeting at Cold Spring Harbor Laboratory, New York entitled "Loss of re-replication control in *S. cerevisiae* results in extensive damage" on September 9th, 2005

The following are reportable outcomes from the prior reporting period:

We have published a manuscript in Molecular Biology of the Cell describing some of the work supported by this grant (Green and Li, 2005).

I presented this work in a talk and a poster at the Nucleic Acids Gordon Conference at Salve Regina University on June 6th, 2004.

I also presented this work at a poster presentation at the Mechanisms of Genomic Integrity Conference in Galway, Ireland on June 22nd, 2004.

Conclusions

I have made significant progress in regards to addressing the specific aims proposed in my initial application entitled, "DNA Damage and Genomic Instability Induced by Inappropriate DNA Re-replication." We have published two papers on which I am a first author describing the results supported by this grant. One (Green et al, 2006) was published during this reporting period and the other (Green and Li, 2005) was published during the previous reporting period. I have also presented this work at three scientific conferences. At two of them (one this period and one last) I was asked to give a talk describing my work.

To maintain genome stability, the entire genome of a eukaryotic cell must be replicated once and only once per cell cycle. In many organisms, multiple overlapping mechanisms block re-replication, but the consequences of deregulating these mechanisms are poorly understood. I have shown that disrupting these controls in the budding yeast *Saccharomyces cerevisiae* rapidly blocks cell proliferation and leads to a significant DNA damage checkpoint response and DNA double strand breaks. These rapid and severe consequences suggest that even limited and sporadic re-replication could threaten the genome with significant damage.

We have also shown that limited re-replication can be induced when two mechanisms that block re-replication are deregulated. This has enabled us to establish a system in which the consequences of re-replication on genome stability can be studied. We have established an assay to study gene duplication events and have shown that re-replication does, in fact, lead to gene duplication events. We have developed a high throughput microarray CGH assay to study sectors arising from these events and have preliminary evidence that re-replication might lead to gene amplification. If confirmed, this would be the first demonstration that re-replication has the potential to lead to heritable genome instability. Since most cancers, breast cancer included, show significant genomic instability, it is critical that we understand the source of such changes to the genome. We have made a great deal of progress in this project period and anticipate that this will continue in the next project period.

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Genome-wide mapping of DNA synthesis in *S. cerevisiae* reveals that mechanisms preventing re-initiation of DNA replication are not redundant

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Running Title: Multiple Nonredundant Blocks to Re-replication

[Key words: DNA replication, rereplication, re-replication, reinitiation, re-initiation, origins, timing, cell cycle, budding yeast, Saccharomyces cerevisiae, microarray, CGH, CDK, genomic instability]

ABSTRACT

To maintain genomic stability, re-initiation of eukaryotic DNA replication within a single cell cycle is blocked by multiple mechanisms that inactivate or remove replication proteins after G1 phase. Consistent with the prevailing notion that these mechanisms are redundant, we previously showed that simultaneous deregulation of three replication proteins, ORC, Cdc6 and Mcm2-7, was necessary to cause detectable bulk re-replication in G2/M phase in *Saccharomyces cerevisiae*. In this study, we used microarray comparative genomic hybridization (CGH) to provide a more comprehensive and detailed analysis of re-replication. This genome-wide analysis suggests that re-initiation in G2/M phase primarily occurs at a subset of both active and latent origins, but is independent of chromosomal determinants that specify the use and timing of these origins in S phase. We demonstrate that re-replication can be induced within S phase, but differs in amount and location from re-replication in G2/M phase, illustrating the dynamic nature of DNA replication controls. Finally, we show that very limited re-replication can be detected by microarray CGH when only two replication proteins are deregulated, suggesting that the mechanisms blocking re-replication are not redundant. Therefore we propose that eukaryotic re-replication at levels below current detection limits may be more prevalent and a greater source of genomic instability than previously appreciated.

INTRODUCTION

Eukaryotic cells must replicate each portion of their genome precisely once per cell cycle to faithfully transmit that genome to succeeding generations. This cell cycle control is enforced at the hundreds to thousands of replication origins where replication is initiated. As part of this regulation, cells must prohibit re-initiation within a single cell cycle at every origin for many successive generations. Even a small or occasional slip in this control will lead to re-replication, which can potentially compromise genome integrity. Hence, the block to re-initiation must be absolutely effective and reliable.

Studies from many labs have led to a model for the block to re-initiation that is based on the division of the initiation event into two mutually exclusive stages (reviewed in (Bell and Dutta, 2002; Diffley, 2004; Machida *et al.*, 2005)). In the first stage, which is restricted to G1 phase, potential origins are selected on chromosomal DNA by assembly of the Origin Recognition Complex (ORC), Cdc6, Cdt1, and the putative replicative helicase, Mcm2-7 into pre-replicative complexes (pre-RCs). In the second stage, which is restricted to S, G2, and M phases, potential origins are activated to initiate DNA replication by two kinases, a cyclin-dependent kinase (CDK) and Cdc7 kinase. Since CDK activity prevents pre-RC assembly in S, G2 and M phases and origins are not activated in G1 phase, passage through the cell cycle is coupled to exactly one round of replication.

Although this model provides a framework for understanding once and only once initiation, it does not explain how the block to re-initiation can be maintained with such high fidelity. This fidelity can be readily incorporated into the model if multiple overlapping mechanisms prevent pre-RC reassembly. In fact, multiple CDK-dependent

inhibitory mechanisms that target pre-RC components have been identified in a number of eukaryotic organisms. In budding and fission yeast, CDKs appear to down regulate ORC through inhibitory phosphorylation of Orc2 and/or Orc6 (Nguyen *et al.*, 2001; Vas *et al.*, 2001) as well as by direct binding to Orc6 (Wilmes *et al.*, 2004). Additionally, CDKs inhibit Cdc6 (or the *S. pombe* ortholog Cdc18) by promoting Cdc6/Cdc18 degradation (Drury *et al.*, 1997; Jallepalli *et al.*, 1997; Elsasser *et al.*, 1999; Drury *et al.*, 2000), by reducing *CDC6* transcription (Moll *et al.*, 1991), and by directly inhibiting Cdc6/Cdc18 through phosphorylation (Jallepalli *et al.*, 1997) or binding (Mimura *et al.*, 2004). Finally, CDKs also promote the nuclear exclusion of Mcm2-7 and Cdt1 in budding yeast (Labib *et al.*, 1999; Nguyen *et al.*, 2000; Tanaka and Diffley, 2002), in part by direct phosphorylation of Mcm3 (Liku *et al.*, 2005). In metazoans, CDKs have been implicated in Orc1 degradation, Cdt1 degradation and Cdc6 nuclear exclusion (reviewed in (Diffley, 2004)). In addition, metazoan cells have a CDK-independent mechanism involving the protein geminin, which binds to Cdt1 and can prevent it from recruiting Mcm2-7 during S, G2, and M phase (reviewed in (Blow and Dutta, 2005)).

Obtaining clear evidence of re-replication within a single cell cycle has generally required the simultaneous disruption of multiple mechanisms, leading to the presumption that these mechanisms are redundant (Diffley, 2004; Blow and Dutta, 2005). In budding yeast, for example, simultaneous deregulation of ORC phosphorylation, Mcm localization, and Cdc6 protein levels was needed to detect re-replication in G2/M phase (Nguyen *et al.*, 2001). Similarly, disruption of several regulatory mechanisms leads to re-replication in fission yeast (Gopalakrishnan *et al.*, 2001; Vas *et al.*, 2001; Yanow *et*

al., 2001) and in *Xenopus* replication extracts (McGarry and Kirschner, 1998; Arias and Walter, 2005; Li and Blow, 2005; Yoshida *et al.*, 2005).

In addition to the issue of mechanistic redundancy, the model for the block to re-replication makes predictions that are best examined by a genome-wide analysis of re-replication. First, the re-replication that is induced by deregulating pre-RC assembly should initiate from the potential replication origins used during normal replication. Re-initiation from a few origins has been observed by 2-dimensional gel electrophoresis in both budding (Nguyen *et al.*, 2001) and fission (Yanow *et al.*, 2001) yeast, but genome-wide mapping of re-initiation sites is needed to confirm this prediction. Second, deregulation of pre-RC reassembly should be able to induce re-replication throughout the period from S to M phase. Although Cdt1 overexpression has been shown to prolong S phase in *Drosophila* embryos (Thomer *et al.*, 2004), direct evidence for re-replication within S phase is still lacking. Finally, full deregulation of pre-RC reassembly should allow more than one round of re-initiation and result in rampant re-replication. So far, precise deregulation of replication proteins has led to at most a doubling of genomic DNA content, suggesting that additional inhibitory mechanisms remain to prevent re-replication. A more comprehensive analysis of where re-replication occurs in the genome may provide clues to how re-replication is still inhibited.

We have developed a more sensitive and comprehensive assay for re-replication by adapting and streamlining previously published microarray-based assays for analyzing DNA replication in budding yeast. With this assay we present evidence that re-initiation occurs primarily at a subset of the potential origins normally established for S phase without being strongly affected by the chromosomal determinants that specify the

efficiency and timing of these origins in S phase. Our studies suggest that the limited re-replication observed may be due in part to the fewer initiation sites used for re-replication compared to S phase. Additionally, our studies indicate that some of the mechanisms preventing re-replication in G2/M phase also operate in S phase but that the block to re-replication in these two phases is not identical. Finally, we demonstrate that re-initiation from as few as a single origin is detectable when fewer mechanisms are disrupted, consistent with the notion that these mechanisms are not redundant but are each actively maintaining the high fidelity of the block to re-replication.

MATERIALS AND METHODS

Plasmids and Strains

All plasmids are described in Table 1, all strains are described in Table 2 and all oligonucleotides are described in Table 3. Supplemental Methods contains detailed description of plasmid and strain construction.

Yeast media, growth and arrest

Cells were grown in YEP, synthetic complete (SC), or synthetic (S broth) medium (Guthrie and Fink, 1990) supplemented with 2% dextrose (wt/vol), 2% galactose (wt/vol), 3% raffinose (wt/vol), or 3% raffinose (wt/vol) + 0.05% dextrose (wt/vol). For S phase experiments cells were grown overnight in SDC (YJL5038) or SDC-Met,Ura (YJL3248 and YJL5834) and arrested in G1 phase with 50 ng/ml alpha factor (all strains were *bar1*) at 30˚C. Cells were released by filtering, washing, and then resuspending in

prewarmed 30˚C YEPD containing 100 µg/ml pronase, 100 mM hydroxyurea, and 15 µg/ml nocodazole.

To obtain reproducible induction of re-replication, cells were inoculated from a fresh unsaturated culture containing 2% dextrose into a culture containing 3% raffinose + 0.05% dextrose and grown for 12-15 h the night before the experiment. The *GAL1* promoter (*pGAL1*) was induced by addition of 2% galactose and the *MET3* promoter (*pMET3*) was repressed by the addition of 2 mM methionine. All experiments were performed at 30˚C except where noted. For induction of re-replication in G2/M phase, cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 2 mM methionine and 15 µg/ml nocodazole. Once arrested (>90% large budded cells), galactose was added to a final concentration of 2%. In experiments with strains containing *cdc7-1,* cells were grown and arrested at 23˚C. These cultures were split after arresting in G2/M phase and either kept at 23˚C or shifted to 35˚C for 1 hour followed by addition of 2% galactose to both cultures

For induction of re-replication during the release from G1 phase into a G2/M phase arrest, cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were arrested with 50 ng/ml alpha factor (all strains were *bar1*). Once arrested (>95% small budded cells), galactose was added to a final concentration of 2% for 30 minutes. Cells were released by filtering, washing, and then resuspending in prewarmed YEPGal + 2 mM methionine, 100 µg/ml pronase, and 15 µg/ml nocodazole. For the induction of re-replication during a release from G1 phase into S phase, cells arrested and released as described above were resuspended in prewarmed YEPGal + 2 mM methionine, 100 µg/ml pronase, and 100 mM hydroxyurea.

Flow cytometry

Cells were fixed and stained with 1 µM Sytox Green (Molecular Probes, Eugene, OR) as previously described (Haase and Lew, 1997).

Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed as described in Green *et al.* (Green and Li, 2005). Probes for *ARS305*, *ARS607* and *ARS1413* were prepared as described in Nguyen *et al.* (Nguyen *et al.*, 2001).

2-D Gel Electrophoresis

Neutral-neutral two-dimensional (2-D) gel analysis was performed essentially as described at http://fangman-brewer.genetics.washington.edu. The DNA preparation described there is a slight modification of the one used in Huberman *et al.* (Huberman *et al.*, 1987). Modifications to the previous protocols can be found in Supplemental Methods.

Microarray Assay

Microarrays containing 12,034 PCR products representing every ORF and intergenic region were prepared essentially as described (DeRisi *et al.*, 1997; Iyer *et al.*, 2001) (see Supplemental Methods). Genomic DNA was prepared, labeled and hybridized as described in Supplemental Methods.

Data analysis

Raw Cy5/Cy3 ratios from scanned arrays were normalized to the DNA content per cell based on the flow cytometry data to determine absolute copy number of each DNA segment. Raw values were then binned and smoothed using Fourier Convolution Smoothing essentially as described (Raghuraman *et al.*, 2001). Peaks in the replication profiles that were both prominent and reproducible among repetitions of an experiment were identified as origins. Details of data analysis (Supplemental Methods) and examples of raw data (Figure S1) are contained in Supplemental Information. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http:://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE4181.

The "experiment variability" was determined using the equation for calculating one standard deviation. Since there were only two DNA preparations used, each of which was hybridized twice, the trials are not truly independent and thus we call these values "experiment variability" rather than standard deviation.

Scatter Plot

For each pro-ARS (Wyrick *et al.*, 2001), the normalized Cy5/Cy3 ratio of that chromosomal locus during replication or re-replication was determined and plotted. See Supplemental Methods for more details.

RESULTS

A simplified microarray CGH assay for DNA replication

We have adapted and streamlined existing microarray assays (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002) to create a rapid and economical genome-wide assay for yeast DNA replication. Our simplified assay uses comparative genomic hybridization (CGH) to directly measure the increase in DNA copy number arising from replication or re-replication. During S phase replication, the copy number of each DNA segment reflects the timing of its replication because the earlier a DNA segment replicates, the greater the proportion of replicating cells containing a duplication of this segment. Origins, which replicate earlier than neighboring regions, can be localized to chromosomal segments where the copy number reaches a local maxima. Thus, use of microarray CGH to monitor copy number changes across the genome can provide a comprehensive view of the location and efficiency/timing of initiation sites during replication and re-replication.

Figure 1A shows a schematic of our microarray CGH replication assay. Genomic DNA from replicating (or re-replicating) and non-replicating cells is purified and differentially labeled with Cy5 and Cy3. The labeled probes are competitively hybridized to a spotted microarray and the raw Cy5/Cy3 values are normalized such that the average ratio corresponds to the DNA content determined by flow cytometry. Data are smoothed and origins are computationally identified by locating prominent and reproducible peaks in smoothed replication profiles.

Before using the microarray CGH assay to study re-replication, we assessed its reproducibility and its ability to identify known replication origins in the S phase of a wild type S288c strain (flow cytometry data in Figure 1C). Figure 1B and Figure S2 show the mean of the smoothed S phase replication profiles from four hybridizations plus or minus the "experiment variability" (see Methods) for chromosome X. The small

variability demonstrates that this technique is highly reproducible. An overlay of our replication profiles with those generated from previously published data (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002) shows considerable agreement in both peak positions, which reflects origin locations, and peak heights, which reflects origin timing/efficiency. When our peak finding algorithm was applied to our profiles, we obtained origin numbers (212) comparable to those obtained by Rhaguraman *et al.* (332) (Raghuraman *et al.*, 2001) and Yabuki *et al.* (260) (Yabuki *et al.*, 2002). Additionally, the alignment of peaks to origins systematically mapped by 2-D gel electrophoresis or ARS plasmid assay was similar to, or better than, published data (Table S1). Together, these data confirm that our streamlined assay is reproducible and accurate.

Re-replication competent mutant has a mostly normal S phase

We have previously demonstrated that simultaneous deregulation of three pre-RC components (ORC, Mcm2-7, and Cdc6) leads to limited re-replication in G2/M phase arrested cells (Nguyen *et al.*, 2001). These initiation proteins were deregulated by mutations that make the proteins refractory to CDK regulation. First, the CDK consensus phosphorylation sites of two subunits of the origin recognition complex, Orc2 and Orc6, were mutated, preventing Cdc28/Cdk1 phosphorylation of these subunits (*orc2-cdk6A, orc6-cdk4A*). Second, two copies of the SV40 nuclear localization signal were fused to *MCM7* (*MCM7-SVNLS*$_2$) to prevent the Cdc28/Cdk1 promoted net nuclear export of the Mcm2-7 complexes. Finally, an extra copy of *CDC6*, containing a partially stabilizing N-terminal deletion, was placed under control of the galactose inducible promoter (*pGAL1-*Δ*ntcdc6*). This strain re-replicates when Δntcdc6 is induced by addition of

galactose and will be referred to as the OMC re-replicating strain in reference to its deregulation of **O**RC, **M**cm2-7, and **C**dc6.

A major concern in any genetic analysis of replication control is the possibility that the mutations deregulating replication proteins also disrupt their replication activity. Such a nonspecific perturbation would complicate any interpretation of the resulting phenotype. We and others have previously reported that Δnt-cdc6 expressed under the *CDC6* promoter retains full replication initiation function (Drury *et al.*, 2000; Nguyen *et al.*, 2001). To determine whether the mutations deregulating Orc2, Orc6, and Mcm7 in the OMC strain also preserve their initiation function, we compared S phase of the OMC strain (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6*), when re-replication was not induced, to S phase of the congenic wild-type A364a strain (*ORC2 ORC6 MCM7 pGAL1*). When cells were harvested at the same point in S phase (Figure 1E), the replication profiles for the two strains showed considerable overlap (Figures 1D, S3 and S4) although ORC and Mcm7 mutations cause subtle alterations in the initiation of DNA replication. Because two wild-type strains of different strain backgrounds show nearly identical replication profiles (Figures S5 and S6), we believe these differences reflect subtle alterations in the initiation activity of the mutant ORC and Mcm2-7. Nonetheless, we conclude that, overall, the mutant ORC and Mcm2-7 proteins in the OMC strain retain most of their normal initiation activity.

Mapping re-initiating origins

A key prediction of the current model for eukaryotic replication control is that pre-RC reassembly and re-initiation should only occur where pre-RCs normally

assemble, i.e., the potential origins or pro-ARSs identified by Wyrick *et al.* (Wyrick *et al.*, 2001). In our previous characterization of re-replication induced at G2/M phase in the OMC strain (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6*), we observed three active S phase origins re-initiating by 2-D gel-electrophoresis (Nguyen *et al.*, 2001). To comprehensively examine this prediction throughout the genome, we performed microarray CGH on the re-replicating DNA from OMC cells. This re-replicating DNA (flow cytometry in Figure 2A) was competitively hybridized against DNA from a congenic non-re-replicating strain that lacks the inducible Δntcdc6 and will be referred to as the OM strain (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1*). Another source of non-re-replicating control DNA is OMC DNA from G1 phase cells, and when this was used, virtually identical results were obtained (data not shown).

The OMC G2/M phase re-replication profiles are shown in Figure 2B and Figure S7. These data confirm that the incomplete re-replication observed by flow cytometry is distributed over all sixteen chromosomes, as was first suggested by their limited entry into the gel during pulsed-field gel electrophoresis (PFGE) ((Nguyen *et al.*, 2001) and Figure 2C). The re-replication profiles also show that individual chromosomes re-replicate very unevenly, with some segments preferentially re-replicating more than others do.

Application of a peak finding algorithm to OMC re-replication profiles identified 106 re-initiating origins. Most of these origins appear to correspond to chromosomal loci that form pre-RCs in G1 phase as more than 80% of the re-initiating origins map to within 10 kb of a pro-ARS identified by Wyrick *et al.* (Wyrick *et al.*, 2001) as sites of pre-RC binding. The mean distance between the OMC re-initiating origins and the

closest Wyrick pro-ARS (Wyrick *et al.*, 2001) is 7.0 kb. This value is highly significant ($p < 5x10^{-8}$) when compared to the mean distances calculated for equivalent numbers of randomly selected chromosomal loci, as a value of 12.3 kb would be expected by chance (Figure S8).

In an accompanying manuscript, Tanny *et al*. (Tanny *et al.*, 2006) have analyzed the re-replication profile of a strain similar to our OMC strain containing the additional perturbation of a mutation of an RXL motif in ORC6 that abrogates CDK binding and results in a slightly increased extent of re-replication. Although both manuscripts use slightly different data analysis and presentation, (our profiles are presented to preserve absolute copy number information at the cost of less distinctive peaks) the re-replication profiles are strikingly similar (compare Figure S7 to Tanny *et al*. (Tanny *et al.*, 2006) Figure S2). Like our results, 80% of the 123 re-replication origins identified by Tanny *et al*. (Tanny *et al.*, 2006) are within 10kb of a Wyrick et al pro-ARS, further supporting the notion that re-replication occurs at normal sites of pre-RC formation. Overlap of origins identified in both studies is considerable, with 64% of the origins in this study within 10kb of an origin in Tanny *et al*. (Tanny *et al.*, 2006) (20% would be expected by chance). This overlap becomes even more striking, 80% overlap (expected value is also 20%), when the top 40 highest peaks in our analysis are compared to peaks identified in Tanny *et al*. (Tanny *et al.*, 2006). Together with our previous confirmation by 2-D gel electrophoresis that ARS305, ARS121, and ARS607 re-initiate (Nguyen *et al.*, 2001), these genomic data suggest that re-initiation primarily occurs at a subset of potential S phase origins.

The efficiency with which these potential origins re-initiate in G2/M phase, however, does not correlate with the efficiency or timing with which they initiate in S phase. For example, only 38% of the active S phase origins re-initiate with enough efficiency to be identified as peaks during re-replication in G2/M phase. Moreover, some regions that normally replicate late in S phase, such as those near the telomeres of chromosome III, re-replicate very efficiently in G2/M phase, apparently from very inefficient or latent S phase origins in those regions. For a systematic comparison of re-replication efficiency versus replication timing of all potential S phase origins, we plotted the re-replication copy number versus the replication copy number for the set of pro-ARSs identified by Wyrick *et al.* (Wyrick *et al.*, 2001) (Figure 2D). The absence of any significant correlation (R^2 of 0.0002) indicates that the efficiency or timing of a replication origin in S phase does not determine its re-replication efficiency during G2/M phase.

Mechanisms that prevent re-replication at G2/M phase also act in S phase

The prevailing model for replication control depicts the prevention of re-replication in S, G2, and M phase as one continuous inhibitory period using a common strategy of preventing pre-RC reassembly. Since CDKs are active throughout this period, the model would predict that mechanisms used by CDKs to regulate replication proteins should prevent re-replication throughout S, G2, and M phase. To determine if CDK regulation of ORC, Mcm2-7, and Cdc6, which prevents re-replication within G2/M phase, also prevents re-replication in S phase, we induced Δntcdc6 in OMC cells (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6*) as they entered S phase.

OMC cells were arrested in G1 phase with alpha factor, and half the cells were harvested to obtain G1 phase DNA. The remaining cells were induced to express Δntcdc6 and then released from the G1 arrest into a low concentration of HU to delay their replication and allow us to collect them in S phase. Flow cytometry indicated that the released cells were harvested while still in S phase with a DNA content of 1.4 C (Figure 3A). The S phase and G1 phase DNA were competitively hybridized against the yeast genomic microarray to generate a combined replication/re-replication profile for S phase (Figure 3B and Figure S9).

Because normal S phase replication can account for an increase in DNA copy number from 1 to 2, only DNA synthesis beyond this copy number can be unequivocally attributed to re-replication. As seen in Figure 3B and Figure S9, many early origins acquired a DNA copy number greater than 2; in some cases reaching values greater than 3. In the same profiles other chromosomal regions had copy numbers significantly below 2, confirming that cells were indeed in the midst of S phase. In fact, early origins re-initiated while forks from their first round of replication were still progressing and before many late origins had fired. Similar re-replication profiles were observed for re-replicating cells synchronously harvested in S phase in the absence of hydroxyurea (data not shown). These findings thus directly establish that mechanisms used to prevent re-replication in G2/M phase also act within S phase.

Cell cycle position can affect the extent and location of re-replication

To determine if the block to re-replication is modulated during progression through the cell cycle, we compared the re-replication profile of OMC cells (*orc2-cdk6A*

*orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6*) that were induced to re-replicate through a complete S phase with the profile associated with re-replication in G2/M phase. To obtain the former profile, both OMC and control OM cells (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1*)) were arrested in G1 phase with alpha factor followed by addition of galactose to induce Δntcdc6 in the OMC strain. Cells were then released from the G1 arrest, allowed to proceed through S phase, and collected at a G2/M arrest 3 hours after the release. DNA prepared from the OMC and OM strains were competitively hybridized to our yeast genomic microarray to obtain a "G1 release" re-replication profile for the OMC cells.

Flow cytometry showed that both the re-replicating OMC and the control OM strain were in the middle of S phase 1 hour after the release (Figure 4A). As expected for actively replicating chromosomes (Hennessy and Botstein, 1991), the chromosomes of these strains were retained in the wells during PFGE (Figure 4B). Two hours after the release, S phase was mostly complete in the control OM strain and its chromosomes reentered the gel during PFGE. In the OMC strain, however, the induction of re-replication prevented chromosomes from reentering the PFGE gel at both 2 and 3 hr timepoints. Because significant re-replication could be induced in OMC cells delayed in S phase, we believe that re-replication during the progression through S phase contributed to the re-replication seen in the G1 release experiment.

Re-replication induced during G1 release of OMC cells was more extensive than re-replication induced in G2/M phase. Despite comparable lengths of induction, flow cytometry reproducibly indicated that the former accumulated a DNA content of 3.2 C while the latter accumulated only 2.7 C (compare 3h time points in Figure 4A to Figure

2A). More extensive re-replication could also be seen by comparing the re-replication profiles induced during the G1 release (Figure 4C and Figure S10) and the G2/M phase arrest (Figure 2B and Figure S7). In general the peaks in the G1 release profiles were taller than the G2/M phase profiles, suggesting that more efficient or more rounds of re-initiation can occur when re-replication is induced during S phase. For example, *ARS305* reached a copy number of 6.6, indicating it re-initiated a second time, as a single round can only generate a maximum copy number of 4. Overall, multiple rounds of re-initiation were observed on more than half of the chromosomes when re-replication was induced during the G1 release. In contrast, multiple rounds of re-initiation occurred at much fewer loci and to a lesser extent when re-replication was induced in G2/M phase.

A peak finding algorithm identified 87 potential re-initiation sites when re-replication was induced during the G1 release experiment. Of these, 85% were located within 10 kb of a Wyrick pro-ARS Wyrick *et al.* (Wyrick *et al.*, 2001). These data suggest that re-replication induced during a G1 release occurs from S phase origins of DNA replication.

In addition to the extent of re-replication, another significant difference between re-replication induced during the G1 release and re-replication induced during G2/M phase was their pattern of origin usage. As discussed above, efficiency of re-replication in G2/M phase was not correlated with origin usage during S phase. In contrast, the efficiency of re-replication induced during the G1 release exhibited a modest positive correlation with S phase origin timing (Figure 4D). Although we cannot rule out an intrinsic difference in the re-initiation efficiency of early versus late origins when re-replication is induced during the G1 release, the simplest explanation for this correlation

is that earlier replicating origins are cleared of pre-RCs earlier, making them available sooner for reassembly of pre-RCs and re-initiation within S phase.

Limited re-replication is detectable with fewer genetic perturbations

Our previous analysis of budding yeast re-replication failed to detect re-replication when only two pre-RC components were deregulated in G2/M phase (Nguyen *et al.*, 2001). This observation is frequently cited as evidence that eukaryotic replication controls are highly redundant. Both the increased sensitivity of the microarray CGH assay and the enhanced re-replication observed during a G1 release provided opportunities to reexamine whether these controls are indeed redundant in budding yeast.

As a first step, we examined an "OC" strain (*orc2-cdk6A orc6-cdk4A pGAL1-*Δ*ntcdc6*), in which only ORC and Cdc6 are deregulated and compared it to a control "O" strain (*orc2-cdk6A orc6-cdk4A GAL1*), where only ORC is deregulated. In accordance with our previous results (Nguyen *et al.*, 2001), induction of Δntcdc6 in G2/M phase generated no significant increase in DNA content by flow cytometry (Figure 5A) or chromosome immobilization during PFGE (Figure 5C). Similarly, microarray CGH of DNA prepared from the OC and O strains after three hours of galactose induction in G2/M phase detected no re-replication on fifteen out of sixteen chromosomes (Figure S11). However, limited re-replication could clearly be observed on both arms of chromosome III (Figure 5E). Thus, the microarray CGH assay can detect re-replication missed by other assays.

We next asked whether we could detect more re-replication in the OC strain by inducing it during a G1 release. In contrast to the results obtained during a G2/M phase

induction, significant re-replication was detected by flow cytometry and PFGE within 2 hours of the G1 release (Figure 5B and Figure 5D). The re-replication profile of the OC strain induced during a G1 release (Figure 5E and Figure S11) showed broad re-replication zones of approximately 200-500 kb in width on all chromosomes. These results, along with the re-replication induced during G2/M phase, establish that deregulating just ORC and Cdc6 is sufficient to induce re-replication and thus these inhibitory mechanisms are not truly redundant. The greater amount of re-replication induced during G1 release versus G2/M arrest underscores the dynamic character of the block to re-replication and, in this case, is likely due to the incomplete expulsion of Mcm proteins from the nucleus during S phase.

Microarray CGH can detect re-replication initiating primarily from a single origin

To further investigate the question of redundancy in replication control, we examined the consequences of deregulating just Mcm2-7 and Cdc6. We were not able to detect re-replication in the "MC" strain (*MCM7-2NLS pGAL1-*Δ*ntcdc6*) whether Δntcdc6 was induced in G2/M phase or during a G1 release (data not shown). Hence, we further deregulated Cdc6 inhibition by mutating the two full CDK consensus phosphorylation sites on Δntcdc6 to generate the MC_{2A} strain (*MCM7-2NLS* Δ*ntcdc6-cdk2A*). These additional mutations increase the stability of Δntcdc6 (Perkins *et al.*, 2001).

Expression of Δntcdc6-cdk2A in the MC_{2A} strain in either G2/M phase or during a G1 release did not cause a detectable increase in DNA content by flow cytometry (Figures 6A and 6B). However, PFGE suggested that chromosome III re-replicated in a small subset of MC_{2A} cells when Δntcdc6-cdk2A was induced under either protocol

(Figure 6C and 6D). Microarray CGH provided definitive evidence that re-replication occurred, in this strain, primarily on the right arm of chromosome III (Figure 6E and Figure S12).

To confirm that the very limited DNA re-replication in the MC_{2A} strain arose from a canonical re-initiation event, we asked whether this re-replication depended on known origins and initiation proteins. Our peak finding algorithm implicated an initiation event at approximately 297 kb, close to *ARS317*, an inefficient S phase origin located at 291 kb. 2-dimensional gel analysis of *ARS317* (Figure 7A) detected bubble arcs, indicative of replication initiation, in the MC_{2A} strain but not the control "M" strain (*MCM7-2NLS pGAL1*). The immediately adjacent origins, *ARS316* and *ARS318*, only displayed fork arcs (data not shown), suggesting that most of the re-replication on the right arm of chromosome III originates from *ARS317*. Deletion of *ARS317*, but not *ARS316* or *ARS318*, in the MC_{2A} strain eliminated the bulk of the re-replication detected by microarray CGH (Figure 7B and data not shown), demonstrating that re-replication initiates primarily from a single S phase origin.

We next asked whether this re-replication is dependent on the essential initiation factor, Cdc7-Dbf4 kinase. Both MC_{2A} and MC_{2A} *cdc7-1* strains were induced to re-replicate in G2/M phase under permissive (23 °C) and restrictive (35 °C) temperatures for the *cdc7-1* allele. Microarray CGH demonstrated that both strains re-replicated to a similar extent at 23ºC (Figure S13), but at 35 °C there was little or no re-replication in the MC_{2A} *cdc7-1* strain (Figure 7C). Together, the dependence on both *ARS317* and Cdc7-

Dbf4 indicates that the very limited re-replication induced in the MC_{2A} strain arises primarily from a single *bona fide* re-initiation event.

DISCUSSION

Use of microarray CGH as a routine genome-wide assay for budding yeast replication.

We have refined previously published genome-wide replication assays for budding yeast and made them more amenable for routine and widespread use in the study of eukaryotic DNA replication. The previous assays required significant effort and cost to generate a single replication profile and were only used to characterize the normal wild-type S phase (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002). We have obtained comparable replication profiles using a streamlined protocol, collection of a single time point and inexpensive spotted microarrays. Thus, it is feasible to use our streamlined assay to examine the genome-wide replication phenotypes associated with many different genotypes or physiological conditions.

Re-initiation induced in G2/M phase largely follows the rules of origin selection, but not the rules of origin activation, that govern S phase replication.

We have taken advantage of our microarray CGH assay to perform a genome wide analysis of eukaryotic re-replication. This comprehensive analysis has allowed us to examine several key tenets of the current model for replication control. One important tenet is that re-initiation that arises from deregulation of ORC, Mcm2-7, and Cdc6 occur from sites of pre-RC formation in S phase. The overall concordance of mapped re-replication origins with pro-ARSs suggests that the re-initiation occurs at sites that

normally assemble pre-RCs for S phase replication. Although current limitations of the resolution of microarray data prevent a precise match of replication and re-replication origins, in the few cases where this has been directly tested by 2-D gel electrophoresis or deletion analysis (Figure 7 and (Nguyen *et al.*, 2001)), we have confirmed that this is, in fact, the case. Thus, the sequence determinants that select potential origins in S phase appear to be conserved during re-replication.

In contrast to the selection of potential origins, the activation of these origins during re-replication in G2/M phase differs considerably from origin activation during replication in S phase. During S phase replication, poorly understood chromosomal determinant specify which potential origins are activated early, which are activated late, and which remain latent. During re-replication in G2/M phase, all three classes are among the 106 origins that re-initiate, and there is no correlation between the time/efficiency pro-ARSs replicate in S phase and the efficiency with which they re-replicate in G2/M phase. These results suggest that the chromosomal determinants governing S phase origin activation are not preserved during G2/M phase re-replication. Such a conclusion is consistent with the finding that the temporal program for origin firing in S phase is lost by G2/M phase and must be reestablished upon entry into each new cell cycle (Raghuraman *et al.*, 1997).

<u>The block to re-replication uses a common fundamental strategy implemented in a dynamic manner across the cell cycle</u>

Another important tenet of the replication control model is that the blocks to re-replication in S, G2, and M phase use the same fundamental strategy of preventing pre-

RC reassembly. Deregulating the mechanisms that prevent this reassembly in any of these cell cycle phases should thus lead to re-replication. Studies in human, *Drosophila* and *C. elegans* that deregulate geminin (Melixetian *et al.*, 2004), Cdt1 (Thomer *et al.*, 2004), and Cul-4 (which stabilizes Cdt1) (Zhong *et al.*, 2003), respectively, have inferred that re-replication can occur within S phase based on evidence of a prolonged S phase. In this study, we directly demonstrate that cells can re-initiate replication at multiple origins while the first round of replication is still ongoing. Thus, we establish that mechanisms used to prevent re-replication in G2/M phase also prevent re-replication within S phase.

Despite sharing common mechanisms to carry out the same fundamental strategy, the block to re-replication in S phase and G2/M phase are not identical. Two differences are readily apparent when comparing cells re-replicating through S phase during a G1 release with cells re-replicating at a G2/M phase arrest. The first difference is the bias toward re-initiation of early origins that is only observed in the G1 release experiment. The simplest explanation for this bias is suggested by the S phase re-replication profiles, which show re-initiation at early origins occurring before late origins have had a chance to fire. These observations suggest that early origins clear their replication pre-RCs sooner and are more available for pre-RC reassembly during S phase, although other explanations for this bias cannot be ruled out.

The second difference between the G1 release and G2/M phase re-replication is that the amount of re-replication induced during the G1 release was greater than the amount induced in G2/M phase in both the OMC and OC strains. This difference can be observed by flow cytometry but is most striking when G1 release and G2/M phase re-replication profiles are compared. There are a growing number of examples of

mechanisms that vary in their efficacy across the cell cycle, such as Cdc6 degradation in budding yeast (Perkins *et al.*, 2001), Cdt1 degradation in *Xenopus* and humans (Nishitani *et al.*, 2004; Arias and Walter, 2005; Li and Blow, 2005; Yoshida *et al.*, 2005), and geminin inhibition in human cells (Ballabeni *et al.*, 2004). Together these results indicate that the block to re-replication is dynamic with the number and relative contribution of regulatory mechanisms implementing the block changing during the cell cycle.

What is limiting re-replication?

A key difference between re-replication and replication in the OMC strain is that a significantly smaller number of origins initiate efficiently during re-replication (106 versus 193). This reduction in origin firing likely contributes to the limited re-replication observed in the OMC strain and suggests that additional mechanisms are still restraining re-initiation. Consistent with both notions, additional mechanisms inhibiting ORC (by CDK binding to Orc6 (Wilmes *et al.*, 2004)) and Cdc6 (by CDK binding to the N-terminus of phosphorylated Cdc6, (Mimura *et al.*, 2004)) have recently been identified in budding yeast. The latter mechanism is already disrupted in the OMC strain because of the N-terminal deletion of Cdc6. Disrupting the former mechanism in the OMC background moderately enhances re-replication, but this re-replication is still restrained (Wilmes *et al.*, 2004; Tanny *et al.*, 2006), suggesting that still more re-replication controls remain to be identified.

The reduced number of re-initiating pro-ARSs, however, may not be the only factor limiting re-replication. Previous work suggests that a single replication fork should be able to replicate 100-200kb (Dershowitz and Newlon, 1993; van Brabant *et al.*,

2001). Our re-replicating profiles show that the amount of DNA synthesis associated with many re-initiating origins is significantly reduced 100-200 kb away from these origins (Figure S7). These data suggest that re-replicating forks may not be able to progress as far as replicating forks, although a more direct analysis of fork movement will be needed to confirm this hypothesis.

Multiple nonredundant mechanisms work in combination to reduce the probability of re-replication.

We previously showed that we could reliably detect G2/M phase re-replication by flow cytometry in the OMC strain when ORC, Mcm2-7, and Cdc6 are deregulated, but not when only two of the three proteins were deregulated (Nguyen *et al.*, 2001). Since then, there have been many other examples where multiple replication controls had to be disrupted to detect re-replication (reviewed in (Diffley, 2004; Blow and Dutta, 2005)). These observations have led to the presumption that the eukaryotic replication controls are redundant. We favor an alternative view that replication controls are not redundant and that disruption of one or a few of controls can lead to low levels of re-replication.

Failure to detect this re-replication has been due to the insensitivity of standard replication assays. In support of the view, the more sensitive microarray CGH assay used in this study was able to detect G2/M phase re-replication in the OC and MC_{2A} strains. We did not detect re-replication when only a single mechanism was disrupted, but we note that the microarray CGH assay has its own detection limits and may have difficulty detecting rare or sporadic replication events. The development of even more sensitive single-cell assays that can detect these rare re-replication events may reveal that the

chance of re-replication occuring is increased when ORC, Mcm2-7, or Cdc6 is individually deregulated.

Our findings support a model in which the block to re-replication is provided by a patchwork of many mechanisms, each of which contributes to a portion of the block by reducing the probability that re-replication will occur within a cell cycle. The combined action of all these mechanisms is needed to reduce the probability to such low levels that re-replication events become exceedingly rare and virtually prohibited. Successive disruption of these mechanisms does not lead to a sudden collapse of the block after a threshold of deregulation is reached, but instead results in a gradual erosion of the block manifested by incrementally higher frequencies and/or levels of limited re-replication. Because all mechanisms contribute in some way to the block, more than one mechanism or combination of mechanisms can be overridden to generate detectable re-replication. Hence, the fact that disruption of a mechanism is sufficient to induce limited re-replication does not make it the critical or dominant mechanism in the block to re-replication.

Levels of re-replication likely to contribute to genomic instability and tumorigenesis may not be detectable by most currently available assays.

Because genomic instability is associated with, and possibly facilitates, tumorigenesis, there has been much interest in understanding the derangements in DNA metabolism and cell cycle control that can cause genomic instability. Re-replication is a potential source of genomic instability both because it produces extra copies of chromosomal segments and because it generates DNA damage and/or replication stress

(Melixetian *et al.*, 2004; Zhu *et al.*, 2004; Archambault *et al.*, 2005; Green and Li, 2005). Re-replication has also been potentially linked to tumorigenesis by the observation that overexpression of Cdt1, which can contribute to re-replication (reviewed in (Blow and Dutta, 2005)), can transform NIH3T3 into tumorigenic cells (Arentson *et al.*, 2002). However, two considerations have raised concerns about the biological relevance of these potential connections. First, if replication controls are highly redundant, the probability that a cell will spontaneously acquire the multiple disruptions needed to induce re-replicate will be extremely small. Second, we and others have shown that cells undergoing overt re-replication experience extensive inviability (Jallepalli *et al.*, 1997; Yanow *et al.*, 2001; Wilmes *et al.*, 2004; Green and Li, 2005) or apoptosis (Vaziri *et al.*, 2003; Thomer *et al.*, 2004), making cell death a more likely outcome than genomic instability or tumorigenesis.

Our results in this study counter the first concern by challenging the concept of redundancy in replication control and showing that very low levels of re-replication can still be observed when fewer controls are disrupted. We also have evidence that lower levels of re-replication induce lower levels of inviability (data not shown), diminishing the second concern. Consequently, we suggest that re-replication at levels well below current detection limits may occur with greater frequency than previously anticipated and that genomic instability may arise from these low, non-lethal levels of re-replication.

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FIGURE LEGENDS

Figure 1 Use of comparative genomic hybridization (CGH) on spotted microarrays to assay DNA replication.

A) Schematic representation of the CGH replication assay. Genomic DNA is purified from non-replicating and replicating cells, differentially labeled with Cy3 and Cy5, and competitively hybridized to a microarray containing 12,034 ORF and intergenic PCR products. Cy5/Cy3 ratios are normalized so that the average ratio of all elements equals the DNA content of the cells (as determined by flow cytometry). Normalized ratios are plotted against chromosomal position and mathematically smoothed to generate a replication profile. In most cases, two hybridizations are performed from each of two independent experiments. The resulting four replication profiles are averaged into one composite profile, and the locations of origins are identified using a peak finding algorithm. Chromosomal regions lacking data of sufficient quality are represented as gaps in the profiles.

B) CGH replication assay described for Figure 1A was performed on YJL5038**,** a wild-type yeast strain in the S288c background. G1 phase genomic DNA was hybridized

against S phase genomic DNA obtained 120 min after cells were released from G1 phase into media containing hydroxyurea (HU). The composite replication profile (blue line) plus and minus the "experiment variability" (light gray band, see Methods) is shown for Chromosome X. Positions of origins annotated in the Saccharomyces Genome Database (SGD, (Balakrishnan)) (red triangles) and the centromere (black circle) are marked along the X-axis. Replication profiles derived from Raghuraman *et al.* (Raghuraman *et al.*, 2001) (violet line) and Yabuki *et al.* (Yabuki *et al.*, 2002) (orange line) are shown for comparison.

C) S phase progression assayed by flow cytometry for experiment described in Figure 1B at the indicated times following release from G1 phase. DNA content of 1.4 C was used to normalize the S288c replication profile.

D) The S phase replication profile of the re-replication competent OMC strain and the congenic wild-type strain are similar. S phase replication profiles were generated for the OMC strain YJL3248 (*MCM7-2NLS orc2-cdk6A orc6-cdk4A pGAL1-*Δ*ntcdc6 pMET3-HA3-CDC20*) and a congenic wild-type A364a strain YJL5834 (*pGAL1*) essentially as described in Figure 1B except S phase cells were harvested, respectively, at 135 min and 180 min after alpha factor release. The S phase replication profile for the OMC strain (green line) and the A364a strain (black line) for chromosome X is shown. SGD annotated origins (red triangles) and the centromere (black circle) are marked along the X-axis.

E) S phase progression assayed by flow cytometry for experiment described in Figure 1D at the indicated times following release from G1 phase. DNA contents of 1.35

C and 1.4 C, respectively, were used to normalize the OMC and A364a replication profiles.

Figure 2 Re-replication induced during G2/M phase when ORC, Mcm2-7 and Cdc6 are deregulated.

A) G2/M phase re-replication in the OMC strain is readily detectable by flow cytometry. The OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6 pMET3-HA3-CDC20)* and the control OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase. Once arrested, galactose was added, which induced re-replication in the OMC strain. Samples were taken for flow cytometry at the indicated points after galactose addition. The DNA content of 2.7 C at 3 hr was used to normalize the OMC re-replication profile in Figure 2B.

B) Genomic DNA was purified from the OMC strain and the control OM strain after 3 hr of galactose induction as described in Figure 2A and competitively hybridized against each other as described in Figure 1A. The OMC G2/M phase re-replication profiles (black lines, right axis), the OMC S phase replication profiles replotted from Figure 1D (gray lines, left axis), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles) and the centromeres (black circles) are shown for chromosomes III, VI, and XIV.

C) Each chromosome participates when OMC cells are induced to re-replicate in G2/M phase. The OMC strain and the control OM strain from the experiment presented

in Figure 2A were harvested for pulsed field gel electrophoresis (PFGE) at the indicated times. Southern blots of the gel were probed with fragments containing ARS305 to detect chromosome III, ARS607 to detect chromosome VI, and ARS1413 to detect chromosome XIV. For each chromosome the Southern signal for both the gel well and the normal chromosomal position are shown.

D) Replication timing does not correlate with efficiency of G2/M phase re-replication in the OMC strain. For each of the pro-ARSs defined by Wyrick *et al.* (Wyrick *et al.*, 2001), the DNA copy number from the OMC G2/M phase re-replication profile in Figure 2B was plotted versus the DNA copy number from the OMC S phase replication profile in Figure 2B. Line represents linear regression of plot.

Figure 3 Deregulation of ORC, Mcm2-7 and Cdc6 can induce re-replication in S phase.

A) Flow cytometry of OMC cells induced to re-replicate in S phase. The OMC strain YJL3249 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6 pMET3-HA3-CDC20*) was arrested in G1 phase, induced to express Δntcdc6 by the addition of galactose, then released from the arrest into media containing HU to delay cells from exiting S phase. At 4 hr the cells were still in S phase with a DNA content of 1.4 C. This value was used to normalize the re-replication profile in 3B.

B) OMC cells can re-initiate and re-replicate within S phase. Genomic DNA was isolated at the 0 hr (G1 phase) and 4 hr (S phase) time points from the OMC strain YJL3249 as described in Figure 3A and competitively hybridized against each other. The resulting profiles shown for chromosomes III and X reflect copy number increases due to

both replication and re-replication. Locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles) and the centromeres (black circles) are plotted along the X-axis.

Figure 4 Re-replication induced upon release from a G1 arrest when ORC, Mcm2-7 and Cdc6 are deregulated.

A) Robust re-replication of OMC cells following G1 release. The OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-*Δ*ntcdc6 pMET3-HA3-CDC20)* and the control OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G1 phase, exposed to galactose to induce Δntcdc6 in the OMC strain, then released from the arrest into G2/M phase. Samples were taken for flow cytometry at the indicated times after release from the alpha factor arrest. The OMC re-replication profile in Figure 4C was normalized to the 3 hr DNA content of 3.2 C.

B) Cells that were induced to re-replicate in Figure 4A were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C.

C) Re-replication profile of the OMC strain following G1 release. Genomic DNA was purified from the OMC strain and the control OM strain 3 hr after G1 release. The two DNA preparations were labeled and competitively hybridized against each other to generate the G1 release re-replication profiles shown for chromosomes III, VI, and XIV. Locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles) and the centromeres (black circles) are plotted along the X-axis.

D) Re-replication induced in the OMC strain following a G1 release is slightly biased toward early replicating pro-ARSs. For each of the pro-ARSs defined by Wyrick *et al.* (Wyrick *et al.*, 2001), the DNA copy number from the OMC G1 release re-replication profile in Figure 4C was plotted versus the DNA copy number from the OMC S phase replication profile in Figure 2B. Line represents linear regression of plot.

Figure 5 Re-replication can be induced when only ORC and Cdc6 are deregulated.

A) Re-replication is undetectable by flow cytometry in OC cells in G2/M phase. The OC strain YJL3240 (*orc2-cdk6A orc6-cdk4A pGAL1-*Δ*ntcdc6 pMET3-HA3-CDC20)* and the control O strain YJL4832 (*orc2-cdk6A orc6-cdk4A pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase and induced with galactose as described in Figure 2A. Samples for flow cytometry were taken at the indicated times after galactose addition. The OC G2/M re-replication profile in Figure 5E was normalized to the 3 hr DNA content of 2.0 C.

B) Significant re-replication can be induced in OC cells during a G1 release. The OC strain and the control O strain were induced with galactose and released from a G1 arrest as described in Figure 4A. Samples for flow cytometry were taken at the indicated times after G1 release. The OC G1 release re-replication profile in Figure 5E was normalized to the 3 hr DNA content of 2.6 C.

C) Re-replication is not readily detected by PFGE in OC cells in G2/M phase. Strains that were induced to re-replicate in Figure 5A were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C.

D) Some but not all copies of each chromosome participate when OC cells are induced to re-replicate in G2/M phase. Strains that were induced to re-replicate in Figure 5B were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C.

E) Cell cycle position significantly affects the extent of re-replication in the OC strain. The OC strain and the control O strain were induced to re-replicate in G2/M phase or during a G1 release as described, respectively, in Figures 5A and 5B. For each induction protocol, OC and O strain genomic DNA were prepared and competitively hybridized against each other. Shown for chromosomes III, VI, and XIV are OC G2/M phase re-replication profiles (black lines), OC G1 release re-replication profiles (gray lines), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles), and the centromeres (black circles).

Figure 6 Re-replication occurs primarily on a single chromosome when Mcm2-7 and Cdc6 are deregulated

A) Re-replication is undetectable by flow cytometry in MC_{2A} cells in G2/M phase. The MC_{2A} strain YJL4489 (*MCM7-NLS pGAL1-Δntcdc6-cdk2A pMET3-HA3-CDC20*) and the control M strain YJL4486 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase and induced with galactose as described in Figure 2A. Samples for flow cytometry were taken at the indicated times after galactose addition. The MC_{2A} G2/M re-replication profile in Figure 6E was normalized to the 3 hr DNA content of 2.0 C.

B) Re-replication is undetectable by flow cytometry in MC_{2A} cells during a G1 release. The MC_{2A} strain and the control M strain were induced with galactose and released from a G1 arrest as described in Figure 4A. Samples for flow cytometry were taken at the indicated times. The MC_{2A} G1 release re-replication profile in Figure 6E was normalized to the 3 hr DNA content of 2.0 C.

C) A portion of the population of chromosome III molecules participate when MC_{2A} cells are induced to re-replicate in G2/M phase. The strains that were induced to re-replicate in Figure 6A were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C.

D) A portion of the population of chromosome III molecules participate when MC_{2A} cells are induced to re-replicate during a G1 release. The strains that were induced to re-replicate in Figure 6B were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C.

E) Re-replication in the MC_{2A} strain occurs primarily on chromosome III. The MC_{2A} strain and the control M strain were induced to re-replicate in G2/M phase or during a G1 release as described, respectively, in Figures 6A and 6B. For each induction protocol, MC_{2A} and M strain genomic DNA were prepared and competitively hybridized against each other. Shown for chromosomes III, VI, and XIV are MC_{2A} G2/M phase re-replication profiles (black lines), MC_{2A} G1 release re-replication profiles (gray lines), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles) and the centromeres (black circles).

Figure 7 The re-replication arising from deregulation of both Mcm2-7 and Cdc6 depends on *ARS317* and Cdc7.

A) Re-initiation bubbles are induced at *ARS317* when MC_{2A} re-replicates in G2/M phase. The MC_{2A} strain YJL4489 (*MCM7-NLS pGAL1-*Δ*ntcdc6-cdk2A pMET3-HA3-CDC20*) and the control M strain YJL4486 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase and induced with galactose as described in Figure 6A. Genomic DNA was purified from each strain at both 0 and 2 hr after induction and subjected to neutral-neutral 2-dimensional gel electrophoresis. Southern blots of the gels were probed with an *ARS317* fragment. Black arrow indicates re-replication bubbles.

B) *ARS317* sequence is required for the bulk of re-replication induced in MC_{2A} cells. The MC_{2A}-Δ*ars317* strain YJL5858 (*MCM7-NLS pGAL1-*Δ*ntcdc6-cdk2A pMET3-HA3-CDC20* Δ*ars317*) and the control M strain YJL4486 were arrested in G2/M phase and induced with galactose for 3 hours as described in Figure 6A. Genomic DNA from the two strains was competitively hybridized against each other to generate the MC_{2A}-Δ*ars317* G2/M phase re-replication profile shown for chromosome III (gray line). The MC_{2A} G2/M phase re-replication profile from Figure 5E is replotted for comparison (black line). The locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles), and the centromere (black circle) are plotted along the X-axis.

C) Cdc7 kinase is required for re-replication induced in MC_{2A} cells. The MC_{2A} strain YJL4489, the congenic MC_{2A}-*cdc7* strain YJL5821 (*MCM7-2NLS pGAL1-*Δ*ntcdc6-2A pMET3-HA3-CDC20 cdc7-1*) and their respective controls, the M strain YJL4486 and the M-*cdc7* strain YJL5816 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20 cdc7-1*) were induced with galactose as described in Figure 6A, except the initial arrest

was performed at 23° C, and the arrested cells were shifted to 35° C for 1 hr, before the addition of galactose. Genomic DNA was isolated 4 hr after galactose addition and competitively hybridized (MC_{2A} versus M and MC_{2A}-*cdc7* versus M-*cdc7*) as described in Figure 1A. Re-replication profiles for the MC_{2A} (black line) and MC_{2A}-*cdc7* (gray line) strains are shown for chromosome III. Locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (gray triangles), and the centromere (black circle) are plotted along the X-axis.

Table 1. Plasmids used in this study

Plasmid	Key Features	Source
pJL737	*ORC6 URA3*	Nguyen *et al.* 2001
pJL806	*pGAL1 URA3*	Nguyen *et al.* 2001
pJL1206	*MCM7-(NLS)2 URA3*	Nguyen *et al.* 2001
pJL1488	*pGAL1-Δntcdc6-cdk2A URA3*	This study
pJL1489	*pGAL1-Δntcdc6 URA3*	Nguyen *et al.* 2001
pKI1260	*MCM7-(svnls3A)2 URA3*	Nguyen *et al.* 2001
pMP933	*ORC2 URA3*	Nguyen *et al.* 2001
YIp22	*pMET3-HA3-CDC20 TRP1*	Uhlmann *et al.* 2000
pFA6a	*KanMX6*	Wach *et al.* 1994
pAG25	*NatMX4*	Goldstein *et al.* 1999
pPP117	*cdc7-1 URA3*	Hollingsworth *et al.* 1992

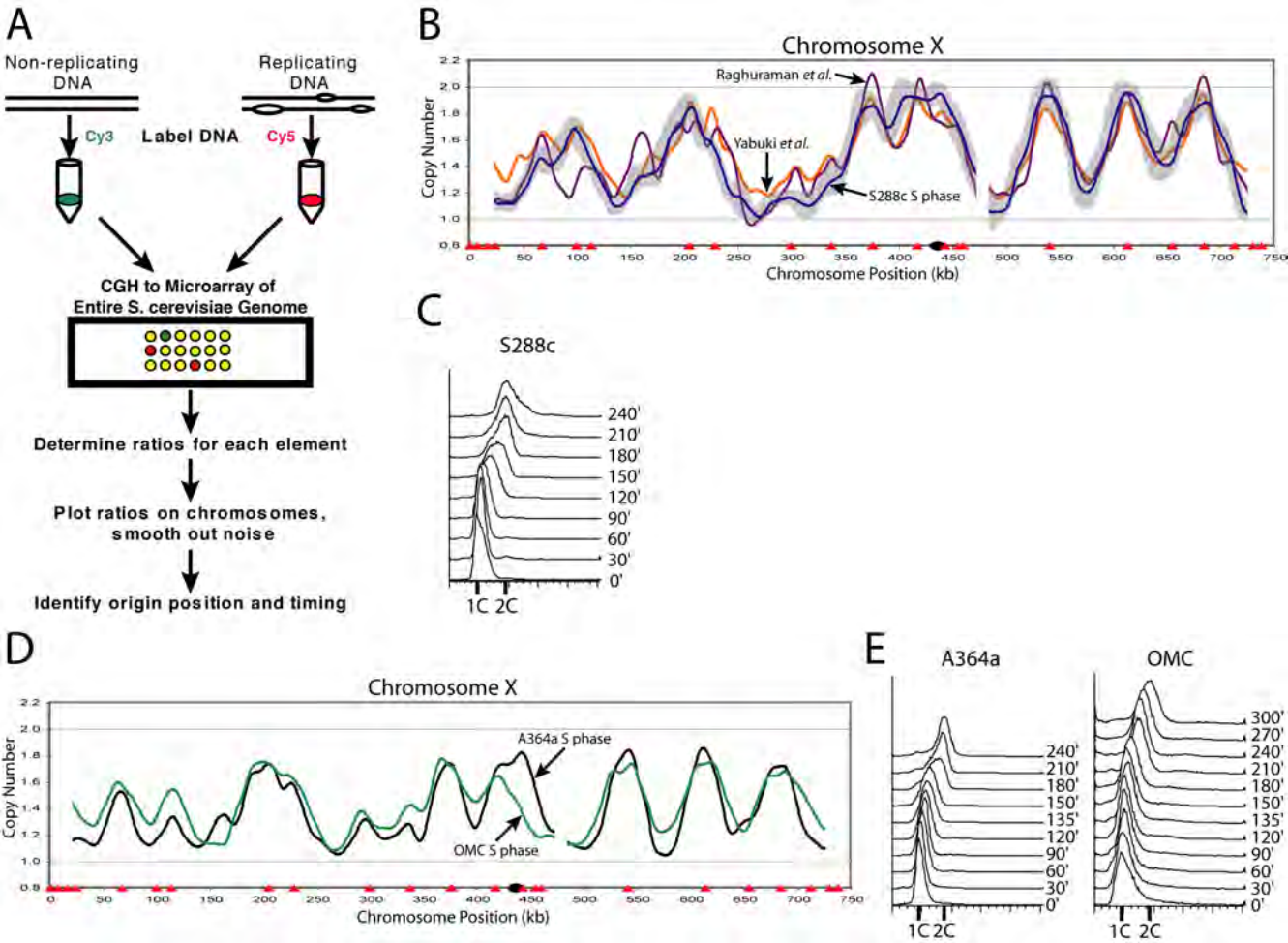
Table 2. Strains used in this study

Strain	Genotype	Source
YJL310	*leu2-3,112 ura3-52 trp1-289 bar1Δ::LEU2*	Detweiter and Li 1998
YJL3244	*orc2-cdk6A orc6-cdk4A leu2 ura3-52::{pGAL1, URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	Nguyen *et al.* 2001
YJL3248	*orc2-cdk6A orc6-cdk4A ura3-52::{pGAL1-Δntcdc6, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	Nguyen *et al.* 2001
YJL3249	*orc2-cdk6A orc6-cdk4A ura3-52::{pGAL1-Δntcdc6, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	This study
YJL4486	*ORC2 ORC6 leu2 ura3-52::{pGAL1, URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	This study
YJL4489	*ORC2 ORC6 ura3-52::{pGAL1-Δntcdc6-cdk2A, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	This study
YJL4832	*orc2-cdk6A orc6-cdk4A ura3-52::{pGAL1, URA3} trp1-289 leu2 ade2 ade3 MCM7-2nls3A bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	This study
YJL3240	*orc2-cdk6A orc6-cdk4A ura3-52::{pGAL1-Δntcdc6, URA3} trp1-289 leu2 ade2 ade3 MCM7-2nls3A bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	This study

YJL5038	*his3Δ::KanMX leu2Δ0 met15Δ0 ura3Δ0 bar1Δ::NatMX4 can1Δ::pMFA1-HIS3::pMFα1-LEU2*	This study
YJL5493	*orc2-cdk6A orc6-cdk4A leu2 ura3-52::{pGAL1, URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*	This study
YJL5834	*ORC2 ORC6 leu2 ura3-52::{pGAL1, URA3} trp1-289 ade2 ade3 MCM7 bar1::LEU2*	This study
YJL5787	*ORC2 ORC6 ura3-52::{pGAL1-Δntcdc6-cdk2A, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1} Δars316::KanMX6*	This study
YJL5858	*ORC2 ORC6 ura3-52::{pGAL1-Δntcdc6-cdk2A, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1} Δars317::KanMX6*	This study
YJL5861	*ORC2 ORC6 ura3-52::{pGAL1-Δntcdc6-cdk2A, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1} Δars318::KanMX4*	This study
YJL5816	*ORC2 ORC6 leu2 ura3-52::{pGAL1, URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1} cdc7-1*	This study
YJL5822	*ORC2 ORC6 ura3-52::{pGAL1-Δntcdc6-cdk2A, URA3} trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1} cdc7-1*	This study

Table 3. Oligonucleotides used in this study

Oligo	Purpose	Sequence
OJL1596	Δ*ARS316*	5'-TTAACTGACAATTCTTTTGAACAAAATTTACACTTCATC AAGAAAGATGCCGGATCCCCGGGTTAATTAA-3'
OJL1597	Δ*ARS316*	5'-TGATGACGAAGGATTCGTTGAAGTTGAATGCACACAAA AAAAGCTTGATACATCGATGAATTCGAGCTCG-3'
OJL1639	Δ*ARS317*	5'-ATTAAACAATGTTTGATTTTTTAAATCGCAATTTAATAC CCGGATCCCCGGGTTAATTAA-3'
OJL1640	Δ*ARS317*	5'-ATTTTTATGGAAGATTAAGCTCATAACTTGGACGGGGAT CCATCGATGAATTCGAGCTCG-3'
OJL1641	Δ*ARS318*	5'-CGATAAAGTTATTATTTAGATTACATGTCACCAACATTT TCGGATCCCCGGGTTAATTAA-3'
OJL1642	Δ*ARS318*	5'-AGAGAAAATAGCTATTTACCTCAACATTTAAAGGTATTA ACATCGATGAATTCGAGCTCG-3'
OJL1607	*ARS317 probe*	5'-ATCGATTATCTGTTTGGCAGG-3'
OJL1608	*ARS317 probe*	5'-GAATTCAAAGAAGTCAATCTTATG-3'
OJL1452	*bar1*Δ	5'-ATTAAAAATGACTATATATTTGATATTTATATGCTATAAA GAAATTGTACTCCAGATTTCCATCGATGAATTCGAGCTCG-3'
OJL1454	*bar1*Δ	5'-AGTGGTTCGTATCGCCTAAAATCATACCAAAATAAAAAGA GTGTCTAGAAGGGTCATATACGGATCCCCGGGTTAATTAA-3'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Replicating DNA
Cy3
Label DNA
Cy5
CGH to Microarray of Entire S. cerevisiae Genome
Determine ratios for each element
Plot ratios on chromosomes, smooth out noise
Identify origin position and timing
B
Chromosome X
Raghuraman et al.
Yabuki et al.
S288c S phase
Copy Number
Chromosome Position (kb)
C
S288c
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210'
180'
150'
120'
90'
60'
30'
0'
1C 2C
D
Chromosome X
A364a S phase
OMC S phase
Copy Number
Chromosome Position (kb)
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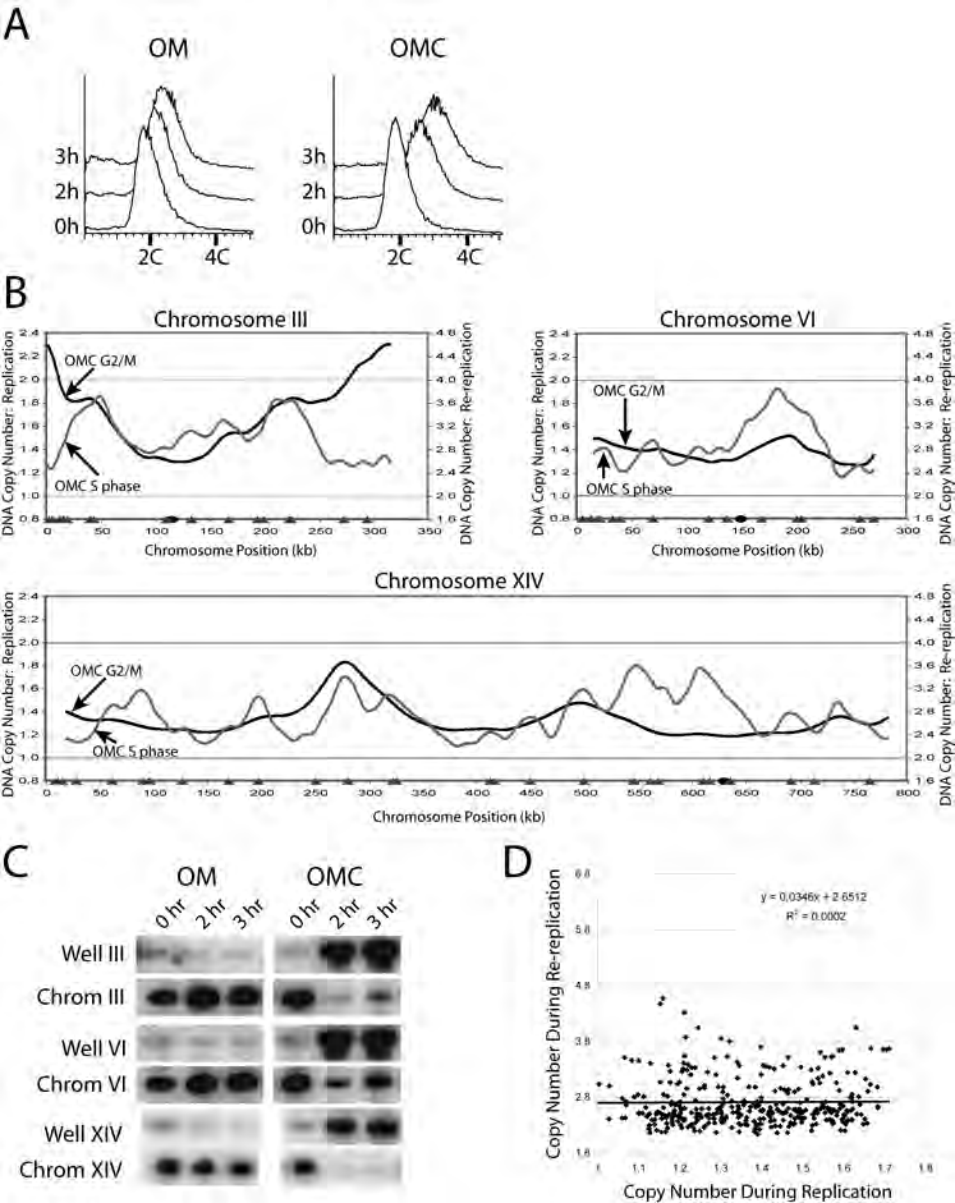
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OMC
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2h
0h
2C
4C
B
Chromosome III
Chromosome VI
Chromosome XIV
OMC G2/M
OMC S phase
DNA Copy Number: Replication
DNA Copy Number: Re-replication
Chromosome Position (kb)
C
OM
OMC
0 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
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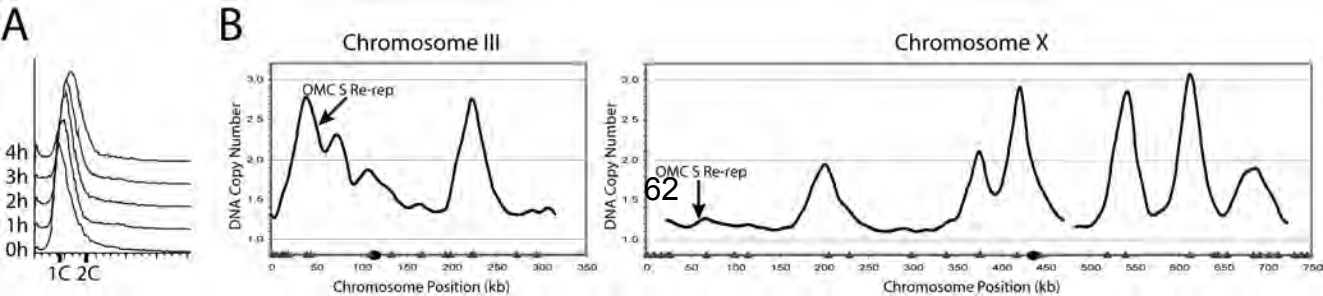
Copy Number During Replication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DNA Copy Number
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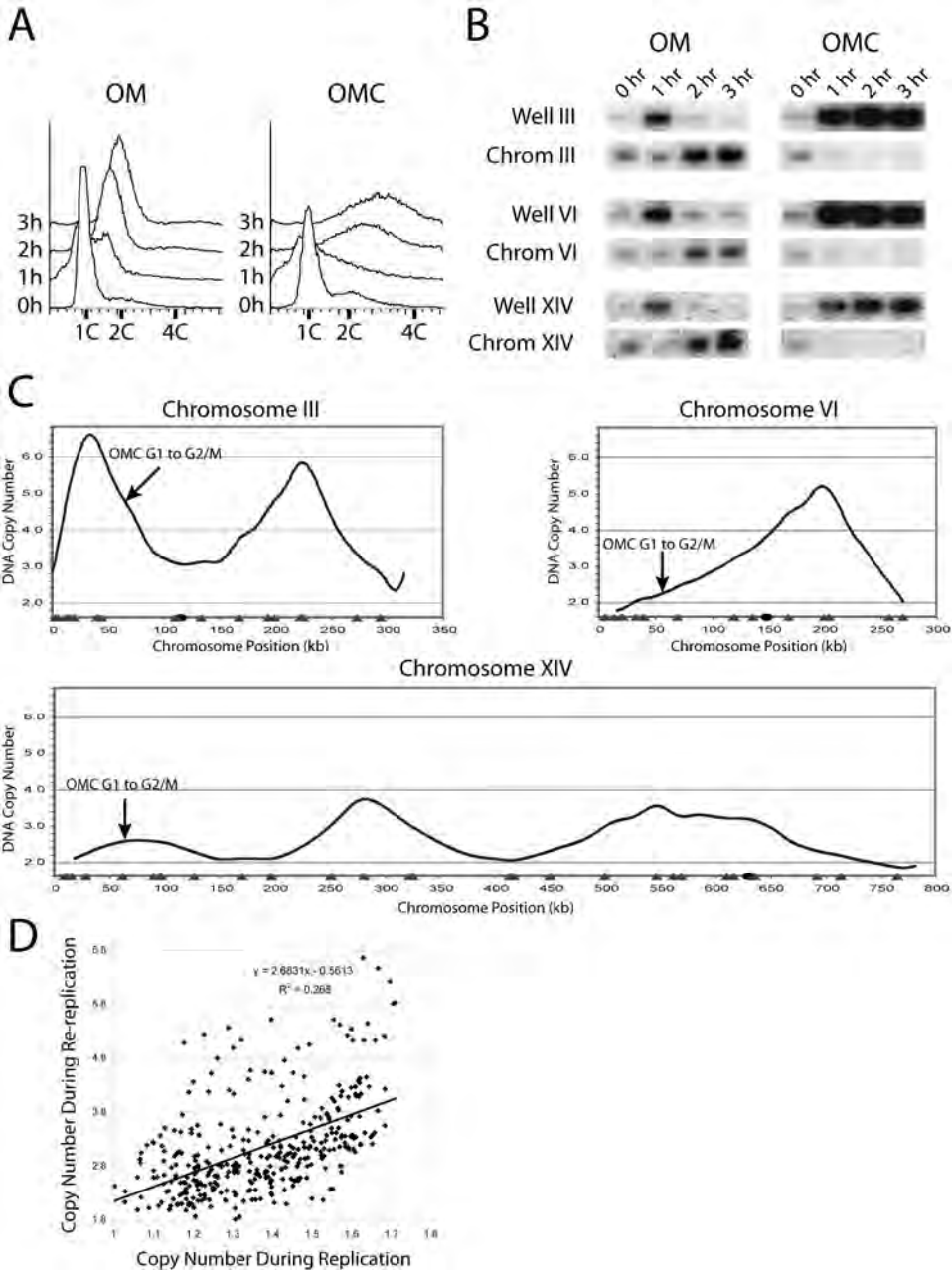
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OMC
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Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
C
Chromosome III
OMC G1 to G2/M
DNA Copy Number
Chromosome Position (kb)
Chromosome VI
OMC G1 to G2/M
DNA Copy Number
Chromosome Position (kb)
Chromosome XIV
OMC G1 to G2/M
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Chromosome Position (kb)
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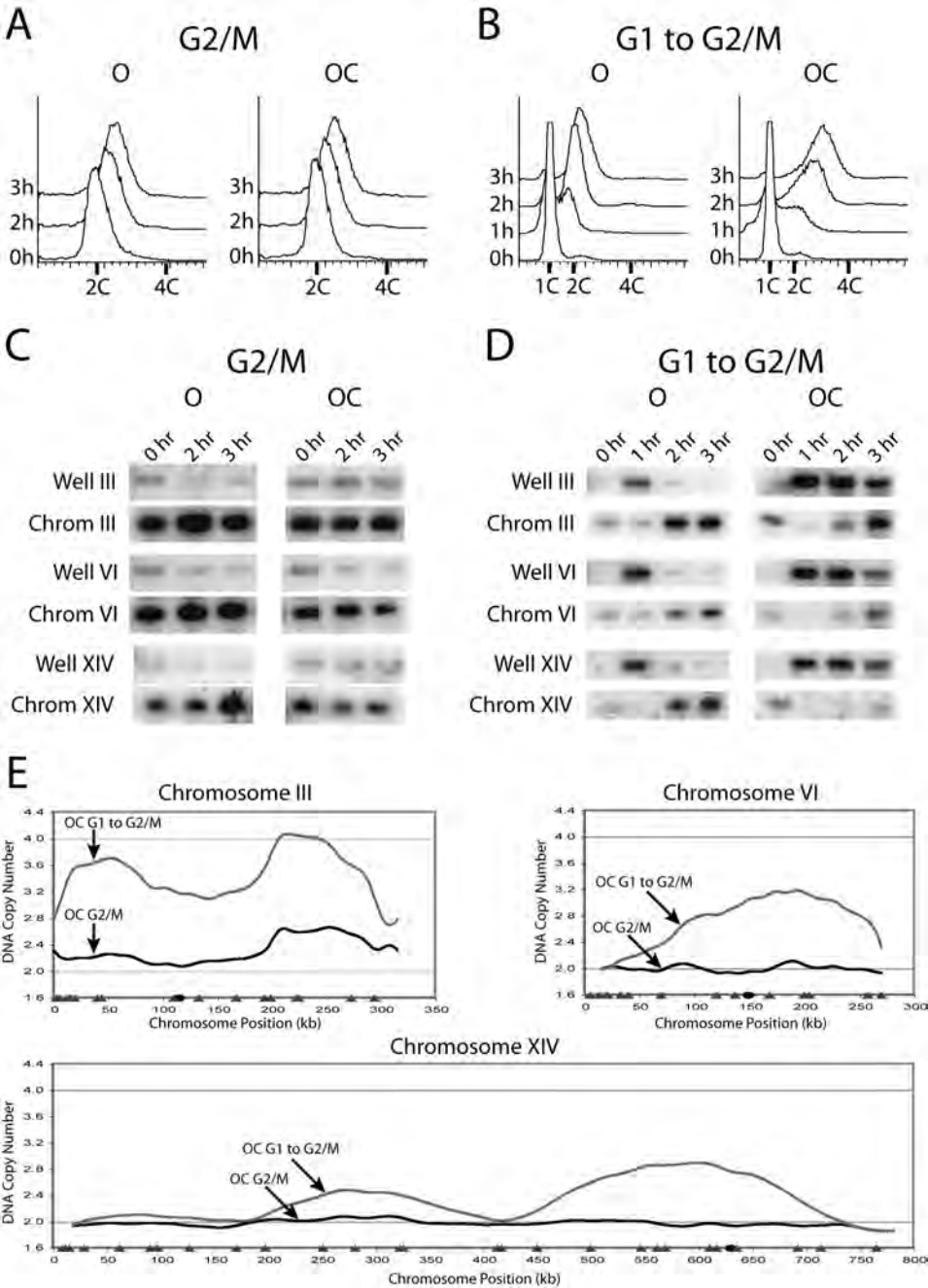
R² = 0.268
Copy Number During Re-replication
Copy Number During Replication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2h
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2C
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2h
1h
0h
1C 2C
4C
C
G2/M
O
OC
0 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
D
G1 to G2/M
O
OC
0 hr
1 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
E
Chromosome III
OC G1 to G2/M
OC G2/M
DNA Copy Number
Chromosome Position (kb)
Chromosome VI
OC G1 to G2/M
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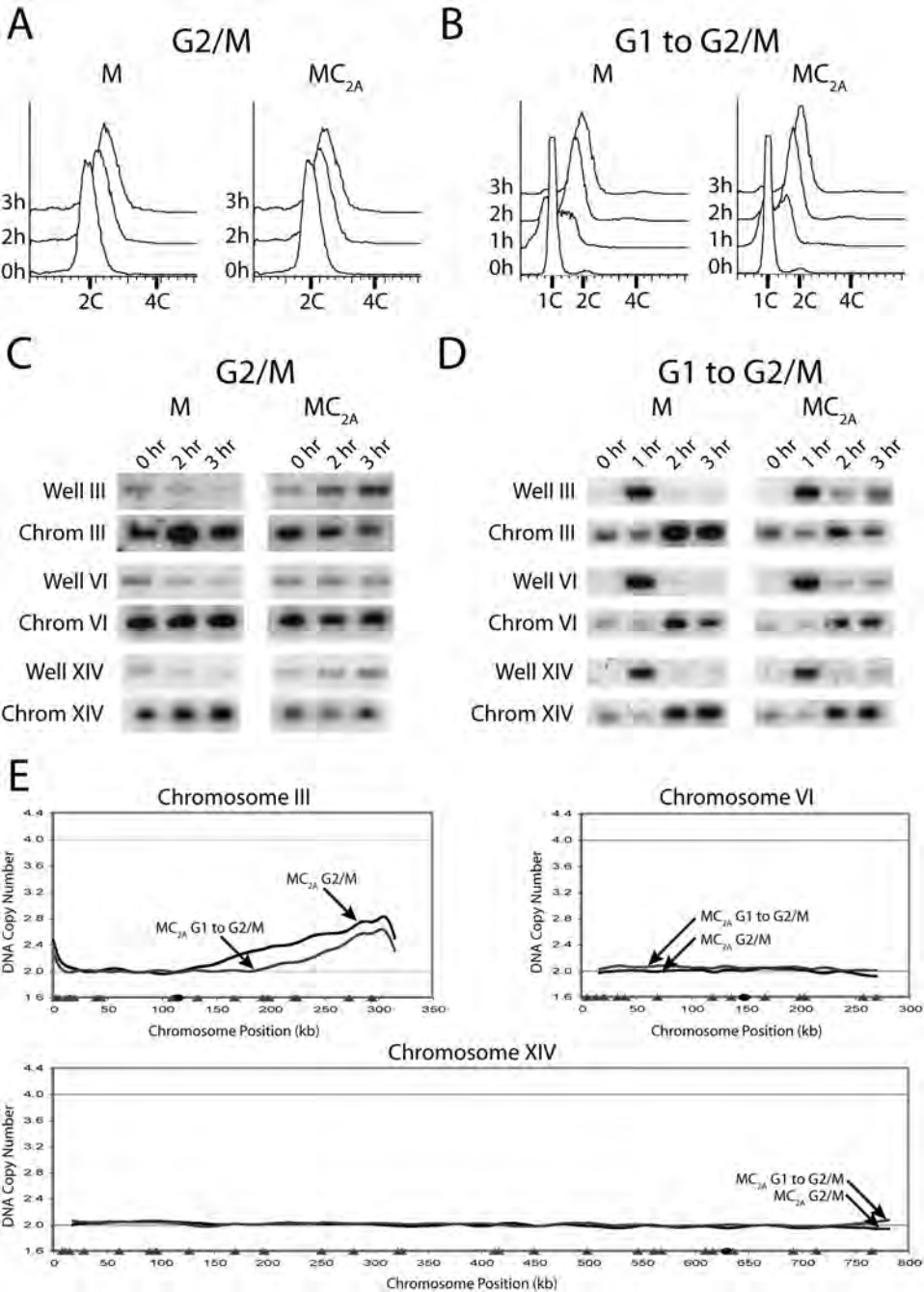
OC G2/M
DNA Copy Number
Chromosome Position (kb)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MC2A
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2C
4C
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MC2A
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2h
1h
0h
1C
2C
4C
C
G2/M
M
MC2A
0 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
D
G1 to G2/M
M
MC2A
0 hr
1 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
E
Chromosome III
DNA Copy Number
MC2A G2/M
MC2A G1 to G2/M
Chromosome Position (kb)
Chromosome VI
DNA Copy Number
MC2A G1 to G2/M
MC2A G2/M
Chromosome Position (kb)
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DNA Copy Number
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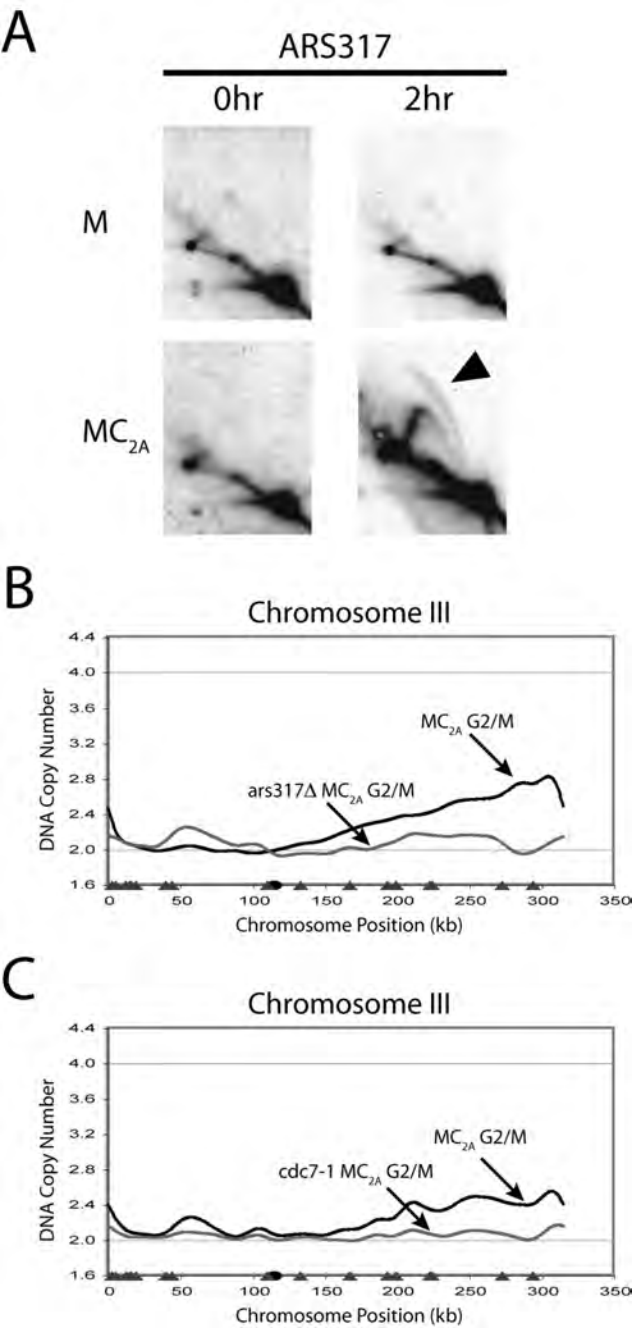
Chromosome Position (kb)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2hr
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MC2A
B
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MC2A G2/M
ars317Δ MC2A G2/M
Chromosome Position (kb)
C
Chromosome III
DNA Copy Number
MC2A G2/M
cdc7-1 MC2A G2/M
Chromosome Position (kb)

Figure 1

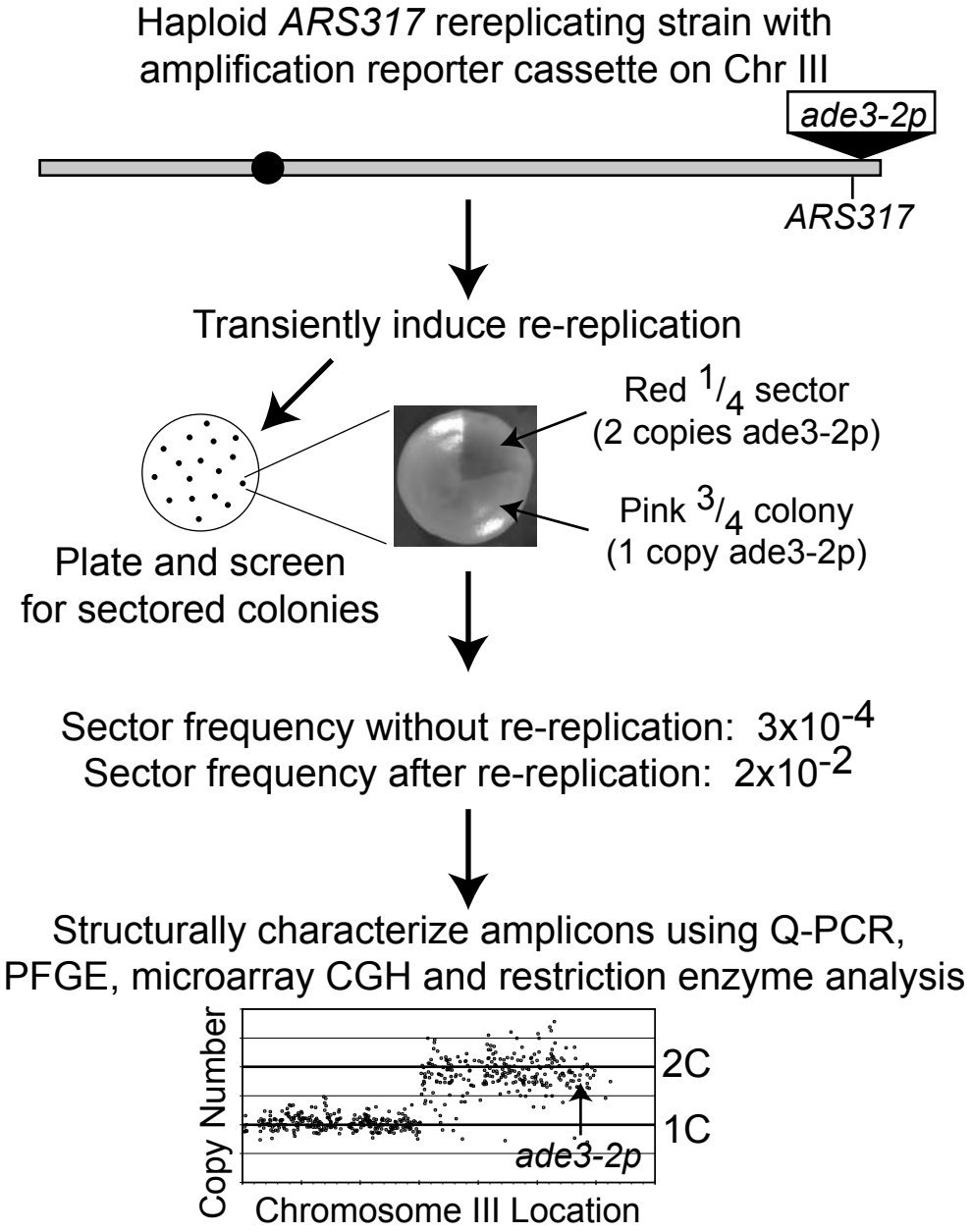

Figure 1 Gene duplication screen. Schematic of assay that we have apadted to study gene duplication events. The hypomorphic ADE3 allele ade3-2p is inserted into the genome near ARS317 in a strain that re-replicates primarily from ARS317. A brief pulse of re-replication is induced and cells are plated on media containing low levels of adenine. Under these conditions, a cell containing one copy of ade3-2p will be pink while one containing two copies will be red. A quarter or half sectored colony is indicative of an event that led to extra copies of ade3-2p in a early cell division after re-replication. Those sectored colonies can be counted and the red cells isolated by streaking. We then characterize the structure of the gene duplication event using numerous assays.

Figure 2

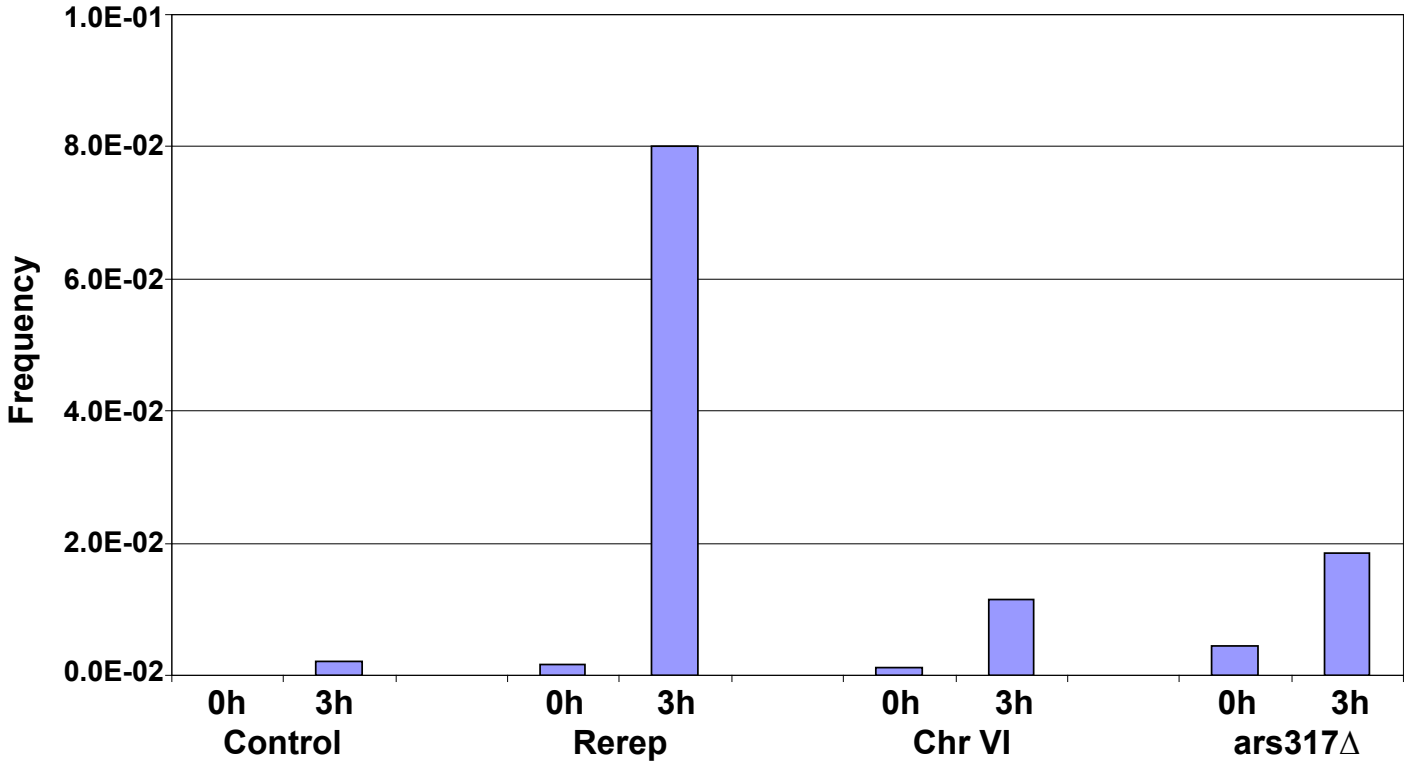

Figure 2 Frequency of gene duplication is stimulated by re-replication. ade3-2p reporter gene was inserted 6 kb from the ARS317 locus in a non-re-replicating control strain (Control), the strain that re-replicates from ARS317 (Rerep), or the re-replicating strain containing a deletion of ARS317 (ars317Δ). The reporter was also inserted in ChrVI in the strain that re-replicates from ARS317 (Chr VI). These strain were arrested in G2/M with nocodazole, then induced to re-replicate by shifting them to galactose for the indicated times, before plating on media with limiting adenine. Parental cells with one copy of ade3-2p are pink, and cells containing two copies of ade3-2p are red. The frequency of colonies containing half, quarter or eighth red sectors are quantified in the table.

Figure 3

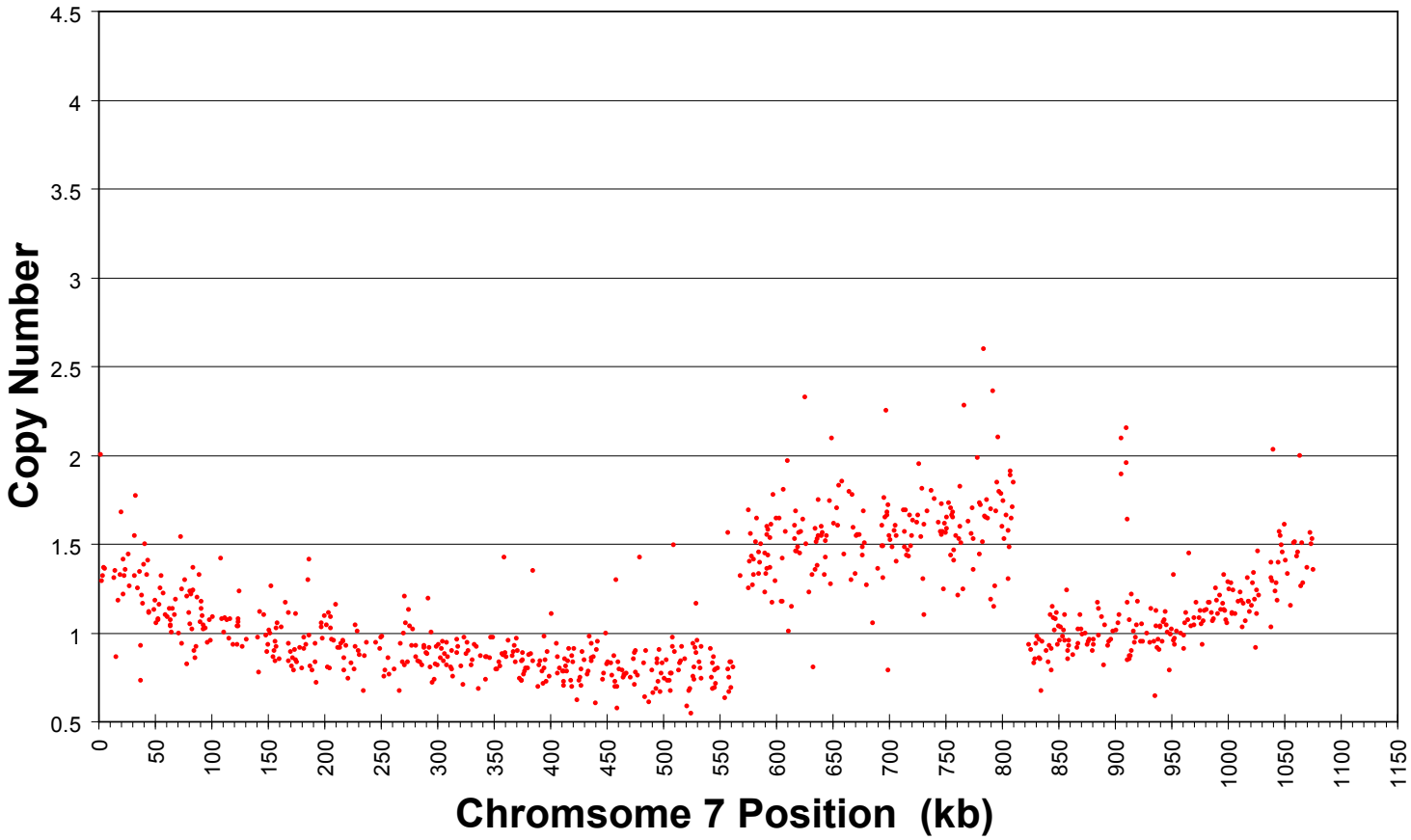

Figure 3 Internal duplication on chromosome 7. Following re-replication of a strain that experienced limited re-replication, one isolate containing a duplication of the ade3-2p reporter on chromosome 3 also displayed a duplication of an internal acentric fragment of chromosome 7. The normalized copy numbers obtained by microarray CGH are shown plotted against the length of chromosome 7. The discontinuites in copy number map close to Ty elements. The slight rise in copy number observed at the ends of the chromosome is due to the high throughput DNA preparation, and disappears when a cleaner DNA preparation is used.